(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 October 2012 (04.10.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/135230 A2

(51) International Patent Classification:
A61K 38/18 (2006.01) *A61K 31/7105* (2006.01)
A61K 38/17 (2006.01) *A61P 17/14* (2006.01)
A61K 48/00 (2006.01) *A61Q 7/00* (2006.01)
A61K 8/64 (2006.01)

(21) International Application Number:
PCT/US2012/030765

(22) International Filing Date:
27 March 2012 (27.03.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/468,544 28 March 2011 (28.03.2011) US
61/474,694 12 April 2011 (12.04.2011) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with declaration under Article 17(2)(a); without abstract; title not checked by the International Searching Authority*

WO 2012/135230 A2

(54) Title: COMPOSITIONS AND METHODS TO MODULATE HAIR GROWTH

(57) Abstract:

COMPOSITIONS AND METHODS TO MODULATE HAIR GROWTH

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119(e) of U.S. Provisional Application Nos. 61/468,544 and 61/474,694, filed March 28, 2011 and April 12, 2011, respectively, the contents of each of which are incorporated by reference into the present disclosure.

STATEMENT OF FEDERAL SUPPORT

[0002] This disclosure was supported by grants from the National Institutes of Health (Grant Nos. RO1-AR42177, AR60306, AR47364 and AR47709). The government has rights in this application.

BACKGROUND

[0003] The skin is the second largest organ in the body. The skin of a mammal is derived from ectoderm and mesoderm layers of an embryo. These two layers give rise to the epidermis and dermis, respectively. The ectoderm and mesoderm layers also give rise to specialized appendages including sensory nerves, sweat glands, and hair follicles.

[0004] Excessive hair (hirsutism) and hair loss (alopecia) are two conditions associated with the skin. Hirsutism is defined as excessive and increased hair growth in locations where the occurrence of terminal hair normally is minimal or absent. It is primarily of cosmetic and psychological concern. The most common form of hair loss (aka alopecia) in men is male pattern baldness (aka androgenic alopecia). In the case of androgenic alopecia, hair loss occurs gradually over several years. It usually starts on the crown of the head and progresses toward the forehead area. In women suffering from alopecia, hair loss occurs in a more dispersed pattern with thinning of the scalp hair and commonly appears following the menopause. Studies to develop a substance for alleviating or treating alopecias of different etiology, particularly a substance for stimulating hair growth or reducing hair loss, have been made from long ago in the cosmetic or pharmaceutical industry field.

[0005] For alopecia, a large number of compounds have been developed as candidate treatments. Examples include 2,4-diamino-6-piperidinopyrimidine-3-oxide (also known

as "minoxidil") and finasteride as disclosed in U.S. Patent No. 4,139,619 and U.S. Patent No. 4,596,812, respectively. A medicament containing minoxidil as an active ingredient is commercially available under the trademark "Rogaine" (Pharmacia & Upjohn Company). A medicament containing finasteride as an active ingredient is commercially available under the trademark "Propecia" (Merck & Co., Inc.). Propecia is a pill for oral administration. Both treatments require continuous application of the compositions to the skin for a long period of time and the success rates are limited.

[0006] Attempts have been made to extracts compositions from natural plants, including medicinal herbs, to be used for the treatment of alopecia. Various extracts of crude drugs, generally known as hair growth compositions, have been used as hair growth stimulants or promoters. Even though some of these hair growth compositions show some effects, the treatments come with some common adverse effects such as skin irritation and unpleasant odor.

[0007] Another method for treatment of alopecia is hair transplantation. This method typically comprises transplanting the natural hair in the scalp area where hair grows to the bald area. Hair transplantation often times is costly, time consuming, painful and only limitedly successful.

[0008] There is a need for the development of a method and a composition to enhance or inhibit hair growth, therefore efficiently treating alopecia or hirsutism, respectively.

SUMMARY OF THE DISCLOSURE

[0009] This disclosure provides methods for facilitating hair growth in a tissue containing a hair follicle comprising administering to the tissue two or more agents selected from the group (a) a Bone Morphogenic Protein (BMP) signaling inhibitor, (b) a secreted frizzled-related protein 4 (Sfrp4) or dickkopf homolog 1 (Dkk1) inhibitor, (c) an NF-κB agonist, (d) a TNF-alpha, IL-1alpha or IL-1beta or an equivalent thereof, or (e) Follistatin, thereby facilitating hair growth.

[0010] Also provided are methods for treating alopecia in a subject having tissue containing a hair follicle, comprising administering to the tissue two or more agents selected from the group (a) a Bone Morphogenic Protein (BMP) signaling inhibitor, (b) a secreted frizzled-related protein 4 (Sfrp4) or dickkopf homolog 1 (Dkk1) inhibitor, (c) an

NF-κB agonist, (d) a TNF-alpha, IL-1alpha or IL-1beta or an equivalent thereof, or (e) Follistatin, thereby treating alopecia in the subject.

BRIEF DESCRIPTION OF THE FIGURES

[0011] **FIG. 1 (A)-(F)** show that regeneration is determined by the density of hair plucking **(A)**. Plucking 200 hairs occupied 1.44π mm^2 (2.4mm in diameter) skin surface area and the hairs regenerate 12 days later. However, when 200 hairs were plucked evenly from 100 mm^2 skin surface area (upper left square), there is no hair regenerate even 30 days later. **(B,C)** show that anagen re-entry occur only when 200 hairs were plucked from the circular surface area which the diameter is lesser than 5mm or the plucking density is higher than $32/\pi$ (hair/mm^2). **(D)** shows that the hair numbers that regenerate are proportional to the surface area where plucked. **Figure 1(E)** shows that when 200 hairs were plucked from circular surface area with diameter of 5mm, the whole area will regenerate simultaneously with 868 hairs regeneration. Couple days later, the surrounding area (6mm in diameter) re-enter anagen, which let the re-grown hairs reach 1263 in total. **(F)** A lot of anagen (right arrow) regenerate from the telogen (left arrow) that wasn't plucked.

[0012] **FIG. 2 (A)-(C)** show genes activated by hair plucking. **(A)** TNF-α, IL-1α, IL-1β and Rel-A showed positive staining in the inter-follicular area from day 2 after plucking (2 days before anagen formation) to day 8. Eda and Wnt5a express restrictedly inside the hair follicle after anagen forms. **(B)** F4/80 + macrophages were increased from day 2 after plucking and **(C)** co-localized with TNF-α.

[0013] **FIG. 3 (A)-(F) and (A')-(F')** show that TNF-α and FGF signaling pathways are involved in plucking induced hair regeneration. **(A, A')** Injection of NF-κB, **(C, C')** FGF, **(D,D')** Ras and **(E, E')** ERK inhibitor but not JNK **(B, B')** inhibitor could delay plucking induced hair regeneration. In contrast, the time period of anagen re-entry after plucking is shortened when TNF70-80 **(F, F')** is injected subcutaneously.

[0014] **FIG. 4 (A)-(D)** show that TNF70-80 stimulates plucking induced hair regeneration. **(A)** Plucking 200 hairs from circular surface area with diameter of 3mm showed TNF-α could express not only below the hairs being plucked but also the hairs not being plucked. **(B)** The expression of BMP-2 is down regulated after 200 hairs are

plucked from circular surface area with diameter of 3mm. **(C)** Subcutaneous injection of TNF70-80 coated beads during refractory telogen period could induce anagen re-entry directly. **(D)** TNF-α knockout mice exhibit a 10 days delay in re-entering anagen after 200 hairs were plucked during refractory telogen phase. Arrows show unplucked hairs and TNF-α expression.

[0015] **FIG. 5** illustrates the mechanism of plucking induced hair regeneration.

[0016] **FIG. 6 (A)-(B)** illustrate a two-dimensional CA model can predict regenerative patterns in a large population of hair stem cells. **(A)** Skin pigmentation patterns result from color changes of many HFs when they collectively cycle through four phases: *P→A→R→C*. Distinct hypothetical activator/inhibitor signaling profiles can be assigned to all four phases. **(B)** A two-dimensional CA model, in which each coded element (*automaton*) represents a single hair follicle (see Fig. 10B), can reproduce regenerative patterns observed in mice: spontaneous initiation, spreading waves, stability and instability of borders. The model can also predict changes in patterns when there are either more inhibitors or activators.

[0017] **FIG. 7 (A)-(F)** show that Wnt signaling plays an activating role in the coordinated regeneration of hair stem cells in a follicle population. **(A)** Wnt7a over-expression in *K14-Wnt7a* mice results in regenerative patterns with shortened *R*-phase, multiple spontaneous initiation centers, fast wave spreading and lack of border stabilization (data not shown). **(B)** Differential expression of key activators and inhibitors in the skin macro-environment (data not shown). **(C, D)** In bead implantation experiments, Wnt3a induces a new regenerative wave **(D)**, whereas Dkk1 disrupts spreading of the existing regenerative wave **(C)** and control BSA has no effect **(D, insert)**. White arrows show directions of anagen spreading waves; dark outlines mark anagen-telogen boundaries. **(E, F)** In *cond-lacZ* WNT-reporter mice, spontaneous WNT-activity (dots) occurs in DPs in *C*-phase but not *R*-phase telogen HFs. Large clusters of WNT-active DPs are very rare (see Table 3). In *cond-lacZ;K14-Wnt7a* mice, 100% of DPs become WNT-active throughout telogen, which induces many new anagen initiation events (white arrows) during *C*-phase (data not shown).

[0018] **FIG. 8 (A)-(E)** present data of model conservation when tested in rabbits. Rabbits exhibit elaborate and rapid regenerative patterns **(A)** that continuously evolve in

time **(F)**. **(B-E)** Rabbits have compound HFs, each containing many separate clusters of K15+ bulge stem cells **(C)** and CD200+ hair germ cells **(D)**. Activation of individual SC clusters within one compound HF is closely coupled **(B, E)** (data not shown).

[0019] **FIG. 9** illustrates a unifying model of coordinated regeneration of hair stem cells. The CA model predicts how simple changes in the relative levels of activators and inhibitors change SC coupling efficiency and modulate duration of $P \rightarrow A \rightarrow R \rightarrow C$ phases (based on Fig. 6(A)). This produces versatile hair regenerative patterns helping animals adapt to different physiological conditions. (Data not shown).

[0020] **FIG. 10 (A)** shows spatio-temporal coupling between follicular stem cell activation and follicular pigmentation. In dormant telogen follicles the epithelial progenitor population consists of bulge stem cells and hair germ cells. At the very beginning of anagen, hair germ progenitor cells are the first to be activated. Their proliferation sustains initial down-growth of the follicle during early anagen. Two days later a portion of bulge stem cells also activate and proliferate (Greco et al. (2009) Cell Stem Cell 4:155-69). About the same time, pigmentation starts within the newly formed bulb of the hair follicle. This results in very close temporal coupling of follicular stem cell activation and the initiation of follicular pigmentation. This can be easily observed at later times through the skin surface.

[0021] **FIG. 10 (B)** illustrates a Cellular Automata framework. Predictive mathematical modeling of the regeneration dynamics among hair stem cell clusters was performed using Cellular Automaton model. In this model, the rectangular patterning field is divided up into a number of square "cells" known as automata. The eight automata surrounding one automaton are defined as its neighbors. In our model, each automaton represents a single hair stem cell cluster (mouse, human) or all hair stem cell clusters of one compound follicle unit (rabbit) as it cycles through the following successive phases (see also Fig. 9): signal propagating (***P***) – non-propagating phases (***A***) - phases refractory (***R***) – competent (***C***) - to such signals. Each automaton remains in a given phase for a variable interval of time after which it moves to the next state. Automata in certain states can interact: ***C***-automata are competent to enter the ***P*** state if triggered to do so. ***P***-automata are able to influence surrounding ***C***-automata to enter ***P*** state (white arrows and thick white borders). ***A***-automata do not influence surrounding automata and cannot be influenced themselves (white stop signs and thick black borders). ***R***-automata unlike ***C***-

automata cannot be induced by ***P***-automata to enter ***P*** state (white stop signs and thick black borders).

[0022] **FIG. 11 (A)-(D)** present predictive modeling of the regeneration dynamics among hair stem cell clusters with balanced activator/inhibitor levels. **(A)** Wave spreading. **(B)** Initiation events. **(C)** Border stability. **(D)** Border instability. Each plot set is labeled with the number of days simulated.

[0023] **FIG. 12** shows CA model simulations of human scalp hair regenerative patterns. Human scalp hair regenerative patterns were simulated using a series of assumptions derived from known human scalp hair regenerative behavior during 1st and 2nd fetal growth cycles (Cutrone and Grimalt (2005) Eur J Pediatr 164:630; Halloy (2000) PNAS 97:8328), as well as during normal adult growth cycles and growth cycles upon alopecia (see **Table 2**). Two types of plots are shown: the right-hand plot shows each automaton state as a shade (*P*, *A*, *R*, *C*) whilst the left-hand plot shows expected follicular pigmentation states as they would appear on the skin (*P* – grey, *A* – black, *R* and *C* – white). Initially all automata are in *C*-phase and several spontaneous initiation sites quickly develop (reflecting first embryonic hair growth cycle; d3.3). The differences between top (fronto-parietal) and bottom (occipital) regions of the field emerge: the top region re-enters *R*-phase (d73.3) and then *C*-phase (d101.7) more quickly and goes through another cycle (reflecting second embryonic hair growth cycle; d105.8). The top region of the field then becomes asynchronous (third postnatal hair growth cycle, d251.7). Meanwhile the bottom region of the field ends its first *A*-phase later (d144.2), re-enters the second synchronized cycle (d246.7) and eventually enters third asynchronous cycle (d410). Eventually, the differences between the top and the bottom domains disappear (d621.7). Towards the end of the simulation, parameters consistent with alopecia are implemented in a section of the top region and there were almost all follicles in *R*-phase.

[0024] **FIG. 13 (A)-(B)** illustrates a unifying model of stem cell regeneration in a large population of hair follicles. **(A)** The spectrum of hair regenerative patterns. **(B)** The spectrum of stem cell topology and hair follicle interactions. During the evolution of hair follicles, epithelial stem cells undergo topological clustering into bulges. Each such cluster can be regulated as one entity, where all stem cells either remain quiescent or become activated relatively synchronously. This allows for the conversion from the continuous renewal mode observed in epidermis to the episodic regeneration mode, such

as that observed in the hair cycle. Stem cell clustering and episodic hair cycling represent evolutionary novelties and enable new ways for the large-scale coordination of regeneration. Each cluster of hair stem cells can become activated by the intrinsic hair cycle clock (Y axis on **B**). Intrinsic activation alone can ensure sufficient levels of hair regeneration if occurs with high probability. This is seen in the adult human scalp, where hair follicles regenerate autonomously based on the intrinsic activation (Y axis on **A**). Hair regeneration based on this mechanism alone can become easily deficient when the probability of intrinsic activation drops, such as upon alopecia. Additionally, this mechanism does not allow for any coordination of regeneration among neighboring hair follicles. It shows that diffusible signaling molecules used for regulating hair stem cell activities within each hair follicle can be co-opted to mediate interactions between neighboring hair follicles. Such signaling couples activation events among many stem cell clusters at once (X axis on **B**). By modulating the strength of intrinsic stem cell activation (Y axis) and the probability of coupled activation (X axis), different animals or different physiological conditions in the same animal can significantly alter the global dynamics of hair regeneration. As the result, versatile hair growth patterns in rabbits, mice, normal and alopecia human scalps can be all explained within the same patterning framework which is based simply on how hair stem cell activities are "managed" **(A)**.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0025] Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference in their entirety into the present disclosure to more fully describe the state of the art to which this disclosure pertains.

Definitions

[0026] The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of tissue culture, immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, e.g., Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edition (1989); Current Protocols In Molecular Biology (F. M. Ausubel, et al. eds., (1987)); the series Methods in Enzymology (Academic Press, Inc.): PCR 2: A Practical Approach

(M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)); Harlow and Lane, eds. (1988) Antibodies, A Laboratory Manual; Harlow and Lane, eds. (1999) Using Antibodies, A Laboratory Manual; and Animal Cell Culture (R.I. Freshney, ed. (1987)).

[0027] All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied (+) or (-) by increments of 1.0 or 0.1, as appropriate. It is to be understood, although not always explicitly stated that all numerical designations are preceded by the term "about". It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

[0028] As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

[0029] As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination for the stated purpose. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this disclosure or process steps to produce a composition or achieve an intended result. Embodiments defined by each of these transition terms are within the scope of this disclosure.

[0030] "Bone Morphogenic Proteins" (BMP) are a group of multifunctional growth factors and cytokines with effects in various tissues. For example, BMPs are known to induce the formation of bone and/or cartilage. Examples of BMP may include, but are not limited to BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 and BMP15.

[0031] "BMP signaling" or "BMP signaling pathway" refers to the enzyme linked receptor protein signaling transduction pathway involving proteins that directly or

indirectly regulate (activate or inhibit) downstream protein activity or gene expression. Examples of molecules involved in the BMP signaling pathways may be found in the public Gene Ontology (GO) database, under GO ID: GO:0030509, accessible at the web page (amigo.geneontology.org/cgi-bin/amigo/term-details.cgi?term=GO:0030509&session_id=5573amigo1226631957), last accessed on November 17, 2008. Without limitation, examples of proteins in the BMP signaling pathway include Activin receptor type-1 (ACVR1, UniProt: Q04771), Activin receptor type-2A (ACVR2A, UniProt: P27037), Activin receptor type-2B (ACVR2B, UniProt: Q13705), BMP1 (UniProt: P13497), BMP2 (UniProt: P12643), BMP3 (UniProt: P12645), BMP4 (UniProt: P12644), BMP5 (UniProt: P22003), BMP6 (UniProt: P22004), BMP7 (UniProt: P18075), BMP8a (UniProt: Q7Z5Y6), BMP8b (UniProt: P34820), BMP10 (UniProt: O95393), BMP15 (UniProt: O95972), Bone morphogenetic protein receptor type-1A (BMPR1A, UniProt: P36894), Bone morphogenetic protein receptor type-1B (BMPR1B, UniProt: O00238), Bone morphogenetic protein receptor type-2 (BMPR2, UniProt: Q13873), Chordin-like protein (CHRDL1, UniProt: Q9BU40), Follistatin-related protein 1 (FSTL1, UniProt: Q12841), Growth/differentiation factor 2 (GDF2, UniProt: Q9UK05), Growth/differentiation factor 6 (GDF6, UniProt: Q6KF10), Growth/differentiation factor 7 (GDF7, UniProt: Q7Z4P5), Gremlin-2 (GREM2, UniProt: Q9H772), RGM domain family member B (RGMB, UniProt: Q6NW40), Ski oncogene (SKI, UniProt: P12755), Mothers against decapentaplegic homolog 4 (SMAD4, UniProt: Q13485), Mothers against decapentaplegic homolog 5 (SMAD5, UniProt: Q99717), Mothers against decapentaplegic homolog 6 (SMAD6, UniProt: O43541), Mothers against decapentaplegic homolog 7 (SMAD7, UniProt: O15105), Mothers against decapentaplegic homolog 9 (SMAD9, UniProt: O15198), E3 ubiquitin-protein ligase SMRF2 (SMURF2, UniProt: Q9HAU4), TGF-beta receptor type III (TGFBR3, UniProt: Q03167), Ubiquitin-conjugating enzyme E2 D1 (UBE2D1, UniProt: P51668), Ubiquitin-conjugating enzyme E2 D3 (UBE2D3, UniProt: P61077) and Zinc finger FYVE domain-containing protein 16 (ZFYVE16, UniProt: Q7Z3T8). Proteins that positively or negatively regulate the BMP signaling, for purpose of this disclosure, are also considered within the meaning of the BMP signaling. Proteins that positively regulate BMP signaling include, but are not limited to, Serine/threonine-protein kinase receptor R3 (ACVRL1, UniProt: P37023) and Endoglin (ENG, UniProt: P17813). Proteins that negatively regulate BMP signaling include, but are not limited to, Chordin (CHRD,

UniProt: Q9H2X0), E3 ubiquitin-protein ligase SMURF1 (SMURF1, UniProt: Q9HCE7), Sclerostin (SOST, UniProt: Q9BQB4) and Brorin (VWC2, UniProt: Q2TAL6). Examples of proteins in the BMP signaling pathway may also include Proprotein convertase subtilisin/kexin type 6 (PCSK6, UniProt: P29122) that regulates BMP signaling.

[0032] Small molecules, polynucleotides, polypeptides that enhance or inhibit BMP signaling exist or can be made with procedures known by those skilled in the art. Yanagita (2009) BioFactors 35(2):113-199 is a review article discussing BMP regulators (incorporated herein by reference). For example, dorsomorphin is a potent small molecule BMP antagonist (Hao et al. (2008) PLoS ONE 3(8):e2904; Yu et al. (2008) Nat. Chem. Biol. 4(1):33-41). Dorsomorphin is currently commercially available from several vendors. Dorsomorphin was reported to selectively inhibit the BMP receptors, type I: ALK2, ALK3 and ALK6 and thus "blocks BMP-mediated SMAD1/5/8 phosphorylation". Dorsomorphin has preferential specificity toward inhibiting BMP versus TGF-beta and activin signaling. In published reports, dorsomorphin is characterized by low toxicity. Dorsomorphin can be delivered into skin to lower macro-environmental BMP signaling and create favorable conditions for hair growth to occur. A unique property of dorsomorphin is that it is a small molecule and is soluble in DMSO. DMSO is known to significantly facilitate trans-dermal delivery of small molecule drugs. This enhancing effect of DMSO on skin penetration can be used in non-invasive method of pharmacological modulation of dermal macro-environment. Treatment procedure thus consists of simply applying liquid form of dorsomorphin in DMSO onto the surface of intact skin. Dorsomorphin in DMSO can be made in form of cream that can be simply rubbed onto intact skin. Small molecule agonist and antagonists for other signaling pathways also exist and can be used to augment or inhibit BMP signaling. Interaction of these small molecules with pathways including, but not limited to, WNT, SHH and FGF will also have direct or indirect impact on BMP signaling thus serve as effective modulator of hair growth via methods disclosed in this disclosure.

[0033] Other types of BMP agonists or antagonists also exist. Yanagita (2009) BioFactors 35(2):113-199 is a review article discussing BMP regulators (incorporated herein by reference). Non-limiting examples include such as noggin, chordin, gremlin, sclerostin and follistatin. Representative sequences for these proteins include UniProt:

Q13253 for noggin, UniProt: Q9H2X0 for chordin, UniProt: O60565 for gremlin, UniProt: Q9BQB4 for sclerostin, and UniProt: P19883 for follistatin. Noggin (UniProt: Q13253), for example, can be produced using methods described in, e.g. McMahon et al. (1998) Genes & Development 12:1438-52.

[0034] In some aspects, an agent that can augment or inhibit BMP signaling is a small molecule agonist or antagonist to a BMP agonist or antagonist. In one aspect, the small molecule is a noggin agonist. In another aspect, the small molecule is a noggin antagonist.

[0035] Examples of agents that can augment or inhibit BMP signaling also include, but are not limited to, polynucleotides that encode BMP proteins, encode polypeptides augmenting or inhibiting BMP signaling, or augmenting or inhibit expression of BMP proteins, or polypeptides augmenting or inhibiting BMP signaling. In some embodiments, the agent is small interference RNA (siRNA) or double strand RNA (dsRNA) that inhibits expression of proteins that augment or inhibit BMP signaling.

[0036] Examples of agents that can augment or inhibit BMP signaling may also include, but are not limited to, an isolated or recombinant BMP protein, or isolated or recombinant polypeptide enhancing or inhibiting BMP signaling. In some aspect, the agent further comprises a pharmaceutically acceptable carrier. In another aspect, the compositions contain carriers that modulate (controlled release) the release of the active agent when administered to a subject in need thereof.

[0037] Examples of polypeptide agents that augment or inhibit BMP signaling may also include, but are not limited to, antibodies or modified antibodies including, but not limited to, blocking fragments of antibodies, that activate, stabilize or inhibit proteins in the BMP signaling pathway or proteins modulating the BMP signaling pathway, thereby augmenting or inhibiting BMP signaling.

[0038] As used herein, the term "modulate" refers to an act by an agent to regulate, to control or to change certain characteristics of the BMP signaling. Examples of the agent may include, but are not limited to, proteins or polypeptides, DNA, RNA, siRNA, dsRNA or other polynucleotides, small molecules. The agent may also mean a temperature change, physical movement or stimulus or any other therapeutical or clinical means that alter the BMP signaling pathway. Without limitation, the object may mean a biochemical

molecule or pathway, a biochemical activity, a medical condition or any other chemical, biochemical, physical or medical aspect of a subject. In one aspect, the term "modulate" means to enhance hair growth on the skin. In another aspect, the term "modulate" means to inhibit hair growth on the skin. In another aspect, the term "modulate" means to positively regulate BMP signaling. In yet another aspect, the term "modulate" means to negatively regulate BMP signaling.

[0039] The terms "facilitate", "augment" and "enhance" as used herein refer to an increase of amount or activity of the target. In one aspect, they refer to activation of the BMP receptors and the downstream signaling, or activation of any downstream signaling without directly activating BMP. In another aspect, they refer to an increase of formation of new hairs on skin, *in vivo* or *in vitro*, or an increase of growth of existing hair.

[0040] The terms "inhibit" or "antagonize" intend mean an decrease of amount or activity of the target. In one aspect, they refer to decrease of activity of the BMP receptors and the downstream signaling, or decrease of any downstream signaling without directly interacting with BMP. In another aspect, they refer to an decrease of formation of new hairs on skin, *in vivo* or *in vitro*, or an reduction of growth of existing hair.

[0041] An "agonist", as used herein, refers to a drug or other chemical that can bind a receptor on a cell to produce a physiologic reaction typical of a naturally occurring substance. The efficacy of an agonist may be positive, causing an increase in the receptor's activity or negative causing a decrease in the receptor's activity.

[0042] An "antagonist" refers to a type of receptor ligand or drug that does not provoke a biological response itself upon binding to the receptor, but blocks or dampens agonist-mediated responses. In pharmacology, antagonists have affinity but no efficacy for their cognate receptors and binding will disrupt the interaction and inhibit the function of an agonist or inverse agonist at receptors. Antagonists mediate their effects by binding to the active site or to allosteric sites on receptors or they may interact at unique binding sites not normally involved in the biological regulation of the receptor's activity. Antagonist activity may be reversible or irreversible depending on the longevity of the antagonist–receptor complex which in turn depends on the nature of antagonist receptor binding. The majority of drug antagonists achieve their potency by competing with endogenous ligands or substrates at structurally defined binding sites on receptors.

[0043] The term "hair growth" intends to include, but not limited to, the formation of new hair or growth of existing hair.

[0044] "Minoxidil" (trade names Rogaine and Regaine; IUPAC name: 6-piperidin-1-ylpyrimidine-2,4-diamine 3-oxide) is a commercially available topical formulation that inhibits hair loss. is a vasodilator medication that is available over the counter for treatment of androgenic alopecia, among other baldness treatments.

[0045] "Finasteride" (IUPAC name *N*-(1,1-dimethylethyl)-3-oxo-(5α,17β)-4-azaandrost-1-ene-17-carboxamide) is a synthetic antiandrogen that acts by inhibiting type II 5-alpha reductase, the enzyme that converts testosterone to dihydrotestosterone (DHT). It is used to treat prostate cancer and is registered in many countries to treat adrogenetic alopecia or male pattern baldness. "Propecia" is a medicament containing finasteride as an active ingredient is commercially available from Merck & Co., Inc.

[0046] An equivalent of a TNF-alpha, IL-1alpha or IL-1beta refers to a polypeptide or modified polypeptide that has at least about 75%, or alternatively at least about 80%, or 85%, or 90%, or 95%, or 98%, or 99% sequence identity to TNF-alpha, IL-1alpha or IL-1beta.

[0047] "Administration", as used herein, refers to the delivery of a medication, such as the agent of the disclosure, which inhibits or augments the BMP signaling, to an appropriate location of the subject, where a therapeutic effect is achieved. Non-limiting examples include oral dosing, intracutaneous injection, direct application to target area proximal areas on the skin, or applied on a patch. Various physical and/or mechanical technologies are available to permit the sustained or immediate topical or transdermal administration of macromolecules (such as, peptides). Such technologies include iontophoresis (see for example Kalia et al., Adv. Drug Del. Rev. 56:619-58, 2004) sonophoresis, needle-less injection, and/or microstructured arrays (sometimes called microneedles; one particular example is the Microstructured Transdermal System (MTS) commercially available from 3M) (see, e.g., Alain et al. (2002) J. Control. Release 81:113-119; Santi et al. (1997) Pharm. Res., 14(1):63-66; Sebastien et al. (1998) J. Pharm. Sci. 87(8):922-925). Methods of making and using arrays of solid microneedles that can be inserted into the skin for transdermal delivery of peptides (such as cyclic CRF antagonists) are provided in Martanto et al. (2004) Pharm. Res. 21:947-52, and Am. Inst.

Chem. Eng. 51:1599-607 (2005). In some examples, the delivery system includes a combination of systems, such as microneedles made of biocompatible and biodegradable polymers (Park et al. (2005) J. Control. Release 104:51-66). Laser systems have also been developed to ablate the stratum corneum from the epidermal layer (Lee et al. (2002) J. Pharm. Sci. 91(7):1613-1626). The laser-ablated regions offer lower resistance to drug (peptide) diffusion than non-ablated skin. In one aspect, administration is topical administration as defined herein.

[0048] "Topical administration" refers to delivery of a medication by application to the skin. Non-limiting examples of topical administration include any methods described under the definition of "administration" pertaining to delivery of a medication to the skin.

[0049] "Interdermal administration" intends delivery of the active ingredient into the dermal layers of the skin, e.g., by use of microneedles or the like.

[0050] "Ablate" or "ablation" of tissue refers to surgical excision or amputation of part of organ or tissue. In one aspect, a mechanical surgical device can be used to excise a layer or part of a layer of the skin such as by tape stripping. In another aspect, laser is used to remove stratum corneum of epidermis to increase the permeability of the skin. The types of surgical devices and procedure, and the type and amount of laser used are known in the art. It should be understood although not always explicitly stated that ablation of tissue may be used prior to treatment as described herein.

[0051] A penetration or permeation enhancer refers to a chemical composition or mechanical/electrical device that can increase the transdermal drug delivery efficiency. In one aspect, a penetration or permeation enhancer is soluble in the formulation and act to reduce the barrier properties of human skin. The list of potential skin permeation enhancers is long, but can be broken down into three general categories: lipid disrupting agents (LDAs), solubility enhancers, and surfactants. LDAs are typically fatty acid-like molecules proposed to fluidize lipids in the human skin membrane. Solubility enhancers act by increasing the maximum concentration of drug in the formulation, thus creating a larger concentration gradient for diffusion. Surfactants are amphiphilic molecules capable of interacting with the polar and lipid groups in the skin (see e.g. Francoeur et al., Potts, Russell O. (1990) Pharm. Res. 7:621-7; U.S. Patent No. 5,503,843).

[0052] A "composition" is intended to mean a combination of active agent, cell or population of cells and another compound or composition, inert (for example, a detectable agent or label or biocompatible scaffold) or active, such as a growth and/or differentiation factor.

[0053] A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active such as a biocompatible scaffold, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

[0054] As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, Remington's Pharm. Sci., 15th Ed. (Mack Publ. Co., Easton (1975)). The term includes carriers that facilitate controlled release of the active agent as well as immediate release.

[0055] For topical use, the pharmaceutically acceptable carrier is suitable for manufacture of creams, ointments, jellies, gels, solutions, suspensions, etc. Such carriers are conventional in the art, e.g., for topical administration with polyethylene glycol (PEG). These formulations may optionally comprise additional pharmaceutically acceptable ingredients such as diluents, stabilizers, and/or adjuvants.

[0056] The pharmaceutically acceptable carrier facilitate immediate or controlled release of the active ingredient.

[0057] A "subject" of diagnosis or treatment is a composition, tissue or an animal, such as a mammal, including a human. Non-human animals subject to diagnosis or treatment include, for example, murine, such as rats, mice, canine, such as dogs, leporids, such as rabbits, bovine, simian, ovine, livestock, sport animals, and pets.

[0058] An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

[0059] A "control" is an alternative subject or sample used in an experiment for comparison purpose. A control can be "positive" or "negative". For example, where the

purpose of the experiment is to determine a correlation of an altered expression level of a gene with a particular phenotype, it is generally preferable to use a positive control (a sample from a subject, carrying such alteration and exhibiting the desired phenotype), and a negative control (a subject or a sample from a subject lacking the altered expression or phenotype). Alternatively, a positive control is an agent exhibiting a desired biological response and a negative control is one that is known not to exhibit the desired biological response.

[0060] As used herein, the terms "treating," "treatment" and the like are used herein to mean obtaining a desired pharmacologic and/or physiologic effect. The effect can be prophylactic in terms of completely or partially preventing a disorder or sign or symptom thereof, and/or can be therapeutic in terms of a partial or complete cure for a disorder and/or adverse effect attributable to the disorder. Examples of "treatment" include but are not limited to: preventing a disorder from occurring in a subject that may be predisposed to a disorder, but has not yet been diagnosed as having it; inhibiting a disorder, i.e., arresting its development; and/or relieving or ameliorating the symptoms of disorder, e.g., alopecia. As is understood by those skilled in the art, "treatment" can include systemic amelioration of the symptoms associated with the pathology and/or a delay in onset of symptoms such as hair loss.

Polynucleotides and Construction, Expression and Delivery

[0061] The terms "nucleic acid", "polynucleotide" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, small interference RNA (siRNA), double strand RNA (dsRNA), ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be

further modified after polymerization, such as by conjugation with a labeling component. The term also refers to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of this disclosure that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0062] A polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. Thus, the term "polynucleotide sequence" is the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

[0063] "Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, or alternatively less than 25% identity, with one of the sequences of the present disclosure.

[0064] A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in Ausubel et al. eds. (2007) Current Protocols in Molecular Biology. Preferably, default parameters are used for alignment. One alignment program is BLAST, using default parameters. In particular, programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations +

SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, last accessed on November 26, 2007. Biologically equivalent polynucleotides are those having the specified percent homology and encoding a polypeptide having the same or similar biological activity.

[0065] The term "a homolog of a nucleic acid" refers to a nucleic acid having a nucleotide sequence having a certain degree of homology with the nucleotide sequence of the nucleic acid or complement thereof. A homolog of a double stranded nucleic acid is intended to include nucleic acids having a nucleotide sequence which has a certain degree of homology with or with the complement thereof. In one aspect, homologs of nucleic acids are capable of hybridizing to the nucleic acid or complement thereof.

[0066] A "gene" refers to a polynucleotide containing at least one open reading frame (ORF) that is capable of encoding a particular polypeptide or protein after being transcribed and translated. Any of the polynucleotide or polypeptide sequences described herein may be used to identify larger fragments or full-length coding sequences of the gene with which they are associated. Methods of isolating larger fragment sequences are known to those of skill in the art.

[0067] The term "express" refers to the production of a gene product.

[0068] As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in an eukaryotic cell.

[0069] The term "encode" as it is applied to polynucleotides refers to a polynucleotide which is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the mRNA for the polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

[0070] "RNA interference" (RNAi) refers to sequence-specific or gene specific suppression of gene expression (protein synthesis) that is mediated by short interfering RNA (siRNA).

[0071] "Short interfering RNA" (siRNA) refers to double-stranded RNA molecules, generally, from about 10 to about 30 nucleotides long that are capable of mediating RNA interference (RNAi)), or 11 nucleotides in length, 12 nucleotides in length, 13 nucleotides in length, 14 nucleotides in length, 15 nucleotides in length, 16 nucleotides in length, 17 nucleotides in length, 18 nucleotides in length, 19 nucleotides in length, 20 nucleotides in length, 21 nucleotides in length, 22 nucleotides in length, 23 nucleotides in length, 24 nucleotides in length, 25 nucleotides in length, 26 nucleotides in length, 27 nucleotides in length, 28 nucleotides in length, or 29 nucleotides in length. As used herein, the term siRNA includes short hairpin RNAs (shRNAs).

[0072] "Double stranded RNA" (dsRNA) refer to double stranded RNA molecules that may be of any length and may be cleaved intracellularly into smaller RNA molecules, such as siRNA. In cells that have a competent interferon response, longer dsRNA, such as those longer than about 30 base pair in length, may trigger the interferon response. In other cells that do not have a competent interferon response, dsRNA may be used to trigger specific RNAi.

[0073] siRNA sequences can be designed by obtaining the target mRNA sequence and determining an appropriate siRNA complementary sequence. siRNAs of the disclosure are designed to interact with a target sequence, meaning they complement a target sequence sufficiently to hybridize to that sequence. An siRNA can be 100% identical to the target sequence. However, homology of the siRNA sequence to the target sequence can be less than 100% as long as the siRNA can hybridize to the target sequence. Thus, for example, the siRNA molecule can be at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to the target sequence or the complement of the target sequence. Therefore, siRNA molecules with insertions, deletions or single point mutations relative to a target may also be used. The generation of several different siRNA sequences per target mRNA is recommended to allow screening for the optimal target sequence. A homology search, such as a BLAST search, should be performed to ensure that the siRNA sequence does not contain homology to any known mammalian gene.

[0074] In general, its preferable that the target sequence be located at least 100-200 nucleotides from the AUG initiation codon and at least 50-100 nucleotides away from the termination codon of the target mRNA (Duxbury (2004) J. Surgical Res. 117:339-344).

[0075] Researchers have determined that certain characteristics are common in siRNA molecules that effectively silence their target gene (Duxbury (2004) J. Surgical Res. 117:339-344; Ui-Tei et al. (2004) Nucl. Acids Res. 32:936–48). As a general guide, siRNAs that include one or more of the following conditions are particularly useful in gene silencing in mammalian cells: GC ratio of between 45-55%, no runs of more than 9 G/C residues, G/C at the 5′ end of the sense strand; A/U at the 5′ end of the antisense strand; and at least 5 A/U residues in the first 7 bases of the 5′ terminal of the antisense strand.

[0076] siRNA are, in general, from about 10 to about 30 nucleotides in length. For example, the siRNA can be 10-30 nucleotides long, 12-28 nucleotides long, 15-25 nucleotides long, 19-23 nucleotides long, or 21-23 nucleotides long. When an siRNA contains two strands of different lengths, the longer of the strands designates the length of the siRNA. In this situation, the unpaired nucleotides of the longer strand would form an overhang.

[0077] The term siRNA includes short hairpin RNAs (shRNAs). shRNAs comprise a single strand of RNA that forms a stem-loop structure, where the stem consists of the complementary sense and antisense strands that comprise a double-stranded siRNA, and the loop is a linker of varying size. The stem structure of shRNAs generally is from about 10 to about 30 nucleotides long. For example, the stem can be 10-30 nucleotides long, 12-28 nucleotides long, 15-25 nucleotides long, 19-23 nucleotides long, or 21-23 nucleotides long.

[0078] Tools to assist siRNA design are readily available to the public. For example, a computer-based siRNA design tool is available on the internet at www.dharmacon.com, last accessed on November 26, 2007.

[0079] dsRNA and siRNA can be synthesized chemically or enzymatically *in vitro* as described in Micura (2002) Agnes Chem. Int. Ed. Emgl. 41:2265–2269; Betz (2003) Promega Notes 85:15–18; and Paddison and Hannon (2002) Cancer Cell. 2:17–23. Chemical synthesis can be performed via manual or automated methods, both of which are well known in the art as described in Micura (2002), *supra*. siRNA can also be endogenously expressed inside the cells in the form of shRNAs as described in Yu et al. (2002) Proc. Natl. Acad. Sci. USA 99:6047–6052; and McManus et al. (2002) RNA

8:842–850. Endogenous expression has been achieved using plasmid-based expression systems using small nuclear RNA promoters, such as RNA polymerase III U6 or H1, or RNA polymerase II U1 as described in Brummelkamp et al. (2002) Science 296:550–553 (2002); and Novarino et al. (2004) J. Neurosci. 24:5322–5330.

[0080] *In vitro* enzymatic dsRNA and siRNA synthesis can be performed using an RNA polymerase mediated process to produce individual sense and antisense strands that are annealed *in vitro* prior to delivery into the cells of choice as describe in Fire et al. (1998) Nature 391:806–811; Donze and Picard (2002) Nucl. Acids Res. 30(10):e46; Yu et al. (2002); and Shim et al. (2002) J. Biol. Chem. 277:30413–30416. Several manufacturers (Promega, Ambion, New England Biolabs, and Stragene) produce transcription kits useful in performing the *in vitro* synthesis.

[0081] *In vitro* synthesis of siRNA can be achieved, for example, by using a pair of short, duplex oligonucleotides that contain T7 RNA polymerase promoters upstream of the sense and antisense RNA sequences as the DNA template. Each oligonucleotide of the duplex is a separate template for the synthesis of one strand of the siRNA. The separate short RNA strands that are synthesized are then annealed to form siRNA as described in Protocols and Applications, Chapter 2: RNA interference, Promega Corporation, (2005).

[0082] *In vitro* synthesis of dsRNA can be achieved, for example, by using a T7 RNA polymerase promoter at the 5′-ends of both DNA target sequence strands. This is accomplished by using separate DNA templates, each containing the target sequence in a different orientation relative to the T7 promoter, transcribed in two separate reactions. The resulting transcripts are mixed and annealed post-transcriptionally. DNA templates used in this reaction can be created by PCR or by using two linearized plasmid templates, each containing the T7 polymerase promoter at a different end of the target sequence. Protocols and Applications, Chapter 2: RNA interference, Promega Corporation, (2005).

[0083] In order to express the proteins described herein, delivery of nucleic acid sequences encoding the gene of interest can be delivered by several techniques. Examples of which include viral technologies (e.g. retroviral vectors, adenovirus vectors, adeno-associated virus vectors, alphavirus vectors and the like) and non-viral technologies (e.g. DNA/liposome complexes, micelles and targeted viral protein-DNA complexes) as described herein. Once inside the cell of interest, expression of the

transgene can be under the control of ubiquitous promoters (e.g. EF-1α) or tissue specific promoters (e.g. keratin 14 promoter (Plikus (2004) J. Pathol. 164:1099-1144; Calcium Calmodulin kinase 2 (CaMKI) promoter, NSE promoter and human Thy-1 promoter). Alternatively expression levels may controlled by use of an inducible promoter system (e.g. Tet on/off promoter) as described in Wiznerowicz et al. (2005) Stem Cells 77:8957-8961.

[0084] Non-limiting examples of promoters include, but are not limited to, the cytomegalovirus (CMV) promoter (Kaplitt et al. (1994) Nat. Genet. 8:148-154), CMV/human β3-globin promoter (Mandel et al. (1998) J. Neurosci. 18:4271-4284), NCX1 promoter, αMHC promoter, MLC2v promoter, GFAP promoter (Xu et al. (2001) Gene Ther., 8:1323-1332), the 1.8-kb neuron-specific enolase (NSE) promoter (Klein et al. (1998) Exp. Neurol. 150:183-194), chicken beta actin (CBA) promoter (Miyazaki (1989) Gene 79:269-277) and the β-glucuronidase (GUSB) promoter (Shipley et al. (1991) Genetics 10:1009-1018), the human serum albumin promoter, the alpha-1-antitrypsin promoter. To improve expression, other regulatory elements may additionally be operably linked to the transgene, such as, e.g., enhancer elements, the Woodchuck Hepatitis Virus Post-Regulatory Element (WPRE) (Donello et al. (1998) J. Virol. 72:5085-5092) or the bovine growth hormone (BGH) polyadenylation site.

[0085] A "gene product" or alternatively a "gene expression product" refers to the amino acid (e.g., peptide or polypeptide) generated when a gene is transcribed and translated.

[0086] A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, micelles biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, or viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

[0087] A polynucleotide can be delivered to a cell or tissue using a gene delivery vehicle. "Gene delivery," "gene transfer," "transducing," and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgene") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, e.g., viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

[0088] A "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo, ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors, alphavirus vectors and the like. Alphavirus vectors, such as Semliki Forest virus-based vectors and Sindbis virus-based vectors, have also been developed for use in gene therapy and immunotherapy. See, Schlesinger and Dubensky (1999) Curr. Opin. Biotechnol. 5:434-439 and Ying et al. (1999) Nat. Med. 5(7):823-827. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, retroviral vector refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

[0089] Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form which integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

[0090] In aspects where gene transfer is mediated by a DNA viral vector, such as an adenovirus (Ad) or adeno-associated virus (AAV), a vector construct refers to the polynucleotide comprising the viral genome or part thereof, and a transgene. Adenoviruses (Ads) are a relatively well characterized, homogenous group of viruses, including over 50 serotypes. See, e.g., International PCT Application No. WO 95/27071. Ads do not require integration into the host cell genome. Recombinant Ad derived vectors, particularly those that reduce the potential for recombination and generation of wild-type virus, have also been constructed. See, International PCT Application Nos. WO 95/00655 and WO 95/11984. Wild-type AAV has high infectivity and specificity integrating into the host cell's genome. See, Hermonat and Muzyczka (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470 and Lebkowski et al. (1988) Mol. Cell. Biol. 8:3988-3996.

[0091] Vectors that contain both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA *in vitro* or *in vivo*, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). In order to optimize expression and/or *in vitro* transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of the clones to eliminate extra, potential inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively, consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

[0092] Gene delivery vehicles also include DNA/liposome complexes, micelles and targeted viral protein-DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this disclosure. To enhance delivery to a cell, the nucleic acid or proteins of this disclosure can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, e.g., a cell surface marker found on stem cells or cardiomyocytes. In addition to the delivery of polynucleotides to a cell or cell population, direct introduction of the proteins described

herein to the cell or cell population can be done by the non-limiting technique of protein transfection, alternatively culturing conditions that can enhance the expression and/or promote the activity of the proteins of this disclosure are other non-limiting techniques.

[0093] The phrase "solid support" refers to non-aqueous surfaces such as "culture plates" "gene chips" or "microarrays." Such gene chips or microarrays can be used for diagnostic and therapeutic purposes by a number of techniques known to one of skill in the art. In one technique, oligonucleotides are arrayed on a gene chip for determining the DNA sequence by the hybridization approach, such as that outlined in U.S. Patent Nos. 6,025,136 and 6,018,041. The polynucleotides of this disclosure can be modified to probes, which in turn can be used for detection of a genetic sequence. Such techniques have been described, for example, in U.S. Patent Nos. 5,968,740 and 5,858,659. A probe also can be affixed to an electrode surface for the electrochemical detection of nucleic acid sequences such as described by Kayem et al. U.S. Patent No. 5,952,172 and by Kelley et al. (1999) Nucleic Acids Res. 27:4830-4837.

[0094] Various "gene chips" or "microarrays" and similar technologies are know in the art. Examples of such include, but are not limited to, LabCard (ACLARA Bio Sciences Inc.); GeneChip (Affymetric, Inc); LabChip (Caliper Technologies Corp); a low-density array with electrochemical sensing (Clinical Micro Sensors); LabCD System (Gamera Bioscience Corp.); Omni Grid (Gene Machines); Q Array (Genetix Ltd.); a high-throughput, automated mass spectrometry systems with liquid-phase expression technology (Gene Trace Systems, Inc.); a thermal jet spotting system (Hewlett Packard Company); Hyseq HyChip (Hyseq, Inc.); BeadArray (Illumina, Inc.); GEM (Incyte Microarray Systems); a high-throughput microarrying system that can dispense from 12 to 64 spots onto multiple glass slides (Intelligent Bio-Instruments); Molecular Biology Workstation and NanoChip (Nanogen, Inc.); a microfluidic glass chip (Orchid biosciences, Inc.); BioChip Arrayer with four PiezoTip piezoelectric drop-on-demand tips (Packard Instruments, Inc.); FlexJet (Rosetta Inpharmatic, Inc.); MALDI-TOF mass spectrometer (Sequnome); ChipMaker 2 and ChipMaker 3 (TeleChem International, Inc.); and GenoSensor (Vysis, Inc.) as identified and described in Heller (2002) Annu. Rev. Biomed. Eng. 4:129-153. Examples of "gene chips" or a "microarrays" are also described in U.S. Patent Publ. Nos.: 2007-0111322, 2007-0099198, 2007-0084997, 2007-0059769 and 2007-0059765 and U.S. Patent Nos.: 7,138,506, 7,070,740, and 6,989,267.

[0095] In one aspect, "gene chips" or "microarrays" containing probes or primers homologous to a polynucleotide, polypeptide or antibody described herein are prepared. A suitable sample is obtained from the patient, extraction of genomic DNA, RNA, protein or any combination thereof is conducted and amplified if necessary. The sample is contacted to the gene chip or microarray panel under conditions suitable for hybridization of the gene(s) or gene product(s) of interest to the probe(s) or primer(s) contained on the gene chip or microarray. The probes or primers may be detectably labeled thereby identifying the gene(s) of interest. Alternatively, a chemical or biological reaction may be used to identify the probes or primers which hybridized with the DNA or RNA of the gene(s) of interest. The genotypes or phenotype of the patient is then determined with the aid of the aforementioned apparatus and methods.

[0096] Other non-limiting examples of a solid phase support include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to a polynucleotide, polypeptide or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. or alternatively polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

[0097] "Eukaryotic cells" comprise all of the life kingdoms except monera. They can be easily distinguished through a membrane-bound nucleus. Animals, plants, fungi, and protists are eukaryotes or organisms whose cells are organized into complex structures by internal membranes and a cytoskeleton. The most characteristic membrane-bound structure is the nucleus. A eukaryotic host, including, for example, yeast, higher plant, insect and mammalian cells, or alternatively from a prokaryotic cells as described above. Non-limiting examples include simian, bovine, porcine, murine, rats, avian, reptilian and human.

[0098] "Prokaryotic cells" that usually lack a nucleus or any other membrane-bound organelles and are divided into two domains, bacteria and archaea. Additionally, instead

of having chromosomal DNA, these cells' genetic information is in a circular loop called a plasmid. Bacterial cells are very small, roughly the size of an animal mitochondrion (about 1-2μm in diameter and 10 μm long). Prokaryotic cells feature three major shapes: rod shaped, spherical, and spiral. Instead of going through elaborate replication processes like eukaryotes, bacterial cells divide by binary fission. Examples include but are not limited to *bacillus* bacteria, *E. coli* bacterium, and *Salmonella* bacterium.

[0099] A "transgenic animal", as used herein, refers to a non-human animal comprising an expression cassette, or a heterologous nucleic acid stably integrated into the animal genome, which expression cassette comprises a polynucleotide encoding a BMP protein, including but not limited to BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 and BMP15, under control of a skin-specific promoter, such as the keratin 14 promoter. The heterologous nucleic acid is introduced into the animal by genetic engineering techniques, such as by trangenic techniques known by those skilled in the art. In another aspect, the expression cassette comprises a polynucleotide encoding a BMP antagonist, such as noggin, chordin, gremlin, sclerostin and follistatin. More details of constructing the expression cassette and transgenic animal are described in Pilkus et al. (2004) Am. J. Pathol. 164:1099-114.

[0100] The term "expression cassette" or "transgenic gene construct" refers to a nucleic acid molecule, e.g., a vector, containing the subject gene, e.g., BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 and BMP15, operably linked in a manner capable of expressing the gene in a host cell. The expression cassette or gene construct can be introduced into a non-human animal cell by nucleic acid-mediated gene transfer by methods known to those skilled in the art.

[0101] In certain aspects, the disclosure is related to an isolated or recombinant BMP protein, polypeptide BMP agonist or antagonist, examples of which are described herein as well as in Yanagita (2009) BioFactors 35(2):113-119. Yanagita (2009) *supra*., reports BMP antagonists and agonists known in the art. Agonists include repulsive guidance molecule (RGMA), DRAGON (RGMB), hemojuvelin, kielin/chordin-like protein (KCP), and Crossveinless 2 (Cv2). Antagonists include chordin, noggin, the eight-membered rings Dan family, the nine-membered ring Tsg family and Crim1. Also encompassed by this disclosure are polypeptides having at least 80% sequence identify, or alternatively 85

% sequence identify, or alternatively 90 % sequence identity, or alternatively 95 % sequence identify, to these polypeptide agonists and antagonists.

[0102] Polypeptides of the disclosure can be prepared by expressing polynucleotides encoding the polypeptide sequences of this disclosure in an appropriate host cell. This can be accomplished by methods of recombinant DNA technology known to those skilled in the art. Accordingly, this disclosure also provides methods for recombinantly producing the polypeptides of this disclosure in a eukaryotic or prokaryotic host cells. The proteins and polypeptides of this disclosure also can be obtained by chemical synthesis using a commercially available automated peptide synthesizer such as those manufactured by Perkin Elmer/Applied Biosystems, Inc., Model 430A or 431A, Foster City, CA, USA. The synthesized protein or polypeptide can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Accordingly, this disclosure also provides a process for chemically synthesizing the proteins of this disclosure by providing the sequence of the protein and reagents, such as amino acids and enzymes and linking together the amino acids in the proper orientation and linear sequence.

[0103] It is known to those skilled in the art that modifications can be made to any peptide to provide it with altered properties. Polypeptides of the disclosure can be modified to include unnatural amino acids. Thus, the peptides may comprise D-amino acids, a combination of D- and L-amino acids, and various "designer" amino acids (*e.g.*, β-methyl amino acids, C-α-methyl amino acids, and N-α-methyl amino acids, etc.) to convey special properties to peptides. Additionally, by assigning specific amino acids at specific coupling steps, peptides with α-helices, β turns, β sheets, α-turns, and cyclic peptides can be generated. Generally, it is believed that α-helical secondary structure or random secondary structure is preferred.

[0104] In a further embodiment, subunits of polypeptides that confer useful chemical and structural properties will be chosen. For example, peptides comprising D-amino acids may be resistant to L-amino acid-specific proteases *in vivo*. Modified compounds with D-amino acids may be synthesized with the amino acids aligned in reverse order to produce the peptides of the disclosure as retro-inverso peptides. In addition, the present disclosure envisions preparing peptides that have better defined structural properties, and the use of peptidomimetics, and peptidomimetic bonds, such as ester bonds, to prepare

peptides with novel properties. In another embodiment, a peptide may be generated that incorporates a reduced peptide bond, *i.e.*, R_1-CH_2NH-R_2, where R_1, and R_2 are amino acid residues or sequences. A reduced peptide bond may be introduced as a dipeptide subunit. Such a molecule would be resistant to peptide bond hydrolysis, *e.g.*, protease activity. Such molecules would provide ligands with unique function and activity, such as extended half-lives *in vivo* due to resistance to metabolic breakdown, or protease activity. Furthermore, it is well known that in certain systems constrained peptides show enhanced functional activity (Hruby (1982) Life Sciences 31:189-199 and Hruby et al. (1990) Biochem J. 268:249-262); the present disclosure provides a method to produce a constrained peptide that incorporates random sequences at all other positions.

[0105] The following non-classical amino acids may be incorporated in the peptides of the disclosure in order to introduce particular conformational motifs: 1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Kazrnierski et al. (1991) J. Am. Chem. Soc. 113:2275-2283); (2S,3S)-methyl-phenylalanine, (2S,3R)- methyl-phenylalanine, (2R,3S)-methyl-phenylalanine and (2R,3R)-methyl-phenylalanine (Kazmierski and Hruby (1991) Tetrahedron Lett. 32(41):5769-5772); 2-aminotetrahydronaphthalene-2- carboxylic acid (Landis (1989) Ph.D. Thesis, University of Arizona); hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Miyake et al. (1989) J. Takeda Res. Labs. 43:53-76) histidine isoquinoline carboxylic acid (Zechel et al. (1991) Int. J. Pep. Protein Res. 38(2):131-138); and HIC (histidine cyclic urea), (Dharanipragada et al. (1993) Int. J. Pep. Protein Res. 42(1):68-77) and (Dharanipragada et al. (1992) Acta. Crystallogr. C. 48:1239-1241).

[0106] The following amino acid analogs and peptidomimetics may be incorporated into a peptide to induce or favor specific secondary structures: LL-Acp (LL-3-amino-2-propenidone-6-carboxylic acid), a β-turn inducing dipeptide analog (Kemp et al. (1985) J. Org. Chem. 50:5834-5838); β-sheet inducing analogs (Kemp et al. (1988) Tetrahedron Lett. 29:5081-5082); β-turn inducing analogs (Kemp et al. (1988) Tetrahedron Lett. 29:5057-5060); α-helix inducing analogs (Kemp et al. (1988) Tetrahedron Lett. 29:4935-4938); α-turn inducing analogs (Kemp et al. (1989) J. Org. Chem. 54:109:115); analogs provided by the following references: Nagai and Sato (1985) Tetrahedron Lett. 26:647-650; and DiMaio et al. (1989) J. Chem. Soc. Perkin Trans. p. 1687; a Gly-Ala turn analog (Kahn et al. (1989) Tetrahedron Lett. 30:2317); amide bond isostere (Clones et al. (1988)

Tetrahedron Lett. 29:3853-3856); tetrazole (Zabrocki et al. (1988) J. Am. Chem. Soc. 110:5875-5880); DTC (Samanen et al. (1990) Int. J. Protein Pep. Res. 35:501:509); and analogs taught in Olson et al. (1990) J. Am. Chem. Sci. 112:323-333 and Garvey et al. (1990) J. Org. Chem. 56:436. Conformationally restricted mimetics of beta turns and beta bulges, and peptides containing them, are described in U.S. Patent No. 5,440,013, issued August 8, 1995 to Kahn.

[0107] It is known to those skilled in the art that modifications can be made to any peptide by substituting one or more amino acids with one or more functionally equivalent amino acids that does not alter the biological function of the peptide. In one aspect, the amino acid that is substituted by an amino acid that possesses similar intrinsic properties including, but not limited to, hydrophobicity, size, or charge. Methods used to determine the appropriate amino acid to be substituted and for which amino acid are know to one of skill in the art. Non-limiting examples include empirical substitution models as described by Dahoff et al. (1978) In Atlas of Protein Sequence and Structure Vol. 5 suppl. 2 (ed. M.O. Dayhoff), pp. 345-352. National Biomedical Research Foundation, Washington DC; PAM matrices including Dayhoff matrices (Dahoff et al. (1978), *supra*, or JTT matrices as described by Jones et al. (1992) Comput. Appl. Biosci. 8:275-282 and Gonnet et al. (1992) Science 256:1443-1145; the empirical model described by Adach and Hasegawa (1996) J. Mol. Evol. 42:459-468; the block substitution matrices (BLOSUM) as described by Henikoff and Henikoff (1992) Proc. Natl. Acad. Sci. USA 89:10915-10919; Poisson models as described by Nei (1987) Molecular Evolutionary Genetics. Columbia University Press, New York.; and the Maximum Likelihood (ML) Method as described by Müller et al. (2002) Mol. Biol. Evol. 19:8-13.

Polypeptide Conjugates

[0108] The polypeptides and polypeptide complexes of the disclosure can be used in a variety of formulations, which may vary depending on the intended use. For example, one or more can be covalently or non-covalently linked (complexed) to various other molecules, the nature of which may vary depending on the particular purpose. For example, a peptide of the disclosure can be covalently or non-covalently complexed to a macromolecular carrier, including, but not limited to, natural and synthetic polymers, proteins, polysaccharides, polypeptides (amino acids), polyvinyl alcohol, polyvinyl pyrrolidone, and lipids. A peptide can be conjugated to a fatty acid, for introduction into

a liposome, see U.S. Patent No. 5,837,249. A peptide of the disclosure can be complexed covalently or non-covalently with a solid support, a variety of which are known in the art and described herein. An antigenic peptide epitope of the disclosure can be associated with an antigen-presenting matrix such as an MHC complex with or without co-stimulatory molecules.

[0109] Examples of protein carriers include, but are not limited to, superantigens, serum albumin, tetanus toxoid, ovalbumin, thyroglobulin, myoglobulin, and immunoglobulin.

[0110] Peptide-protein carrier polymers may be formed using conventional cross-linking agents such as carbodimides. Examples of carbodimides are 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide (CMC), 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC) and 1-ethyl-3-(4-azonia-44-dimethylpentyl) carbodiimide.

[0111] Examples of other suitable cross-linking agents are cyanogen bromide, glutaraldehyde and succinic anhydride. In general, any of a number of homo-bifunctional agents including a homo-bifunctional aldehyde, a homo-bifunctional epoxide, a homo-bifunctional imido-ester, a homo-bifunctional N-hydroxysuccinimide ester, a homo-bifunctional maleimide, a homo-bifunctional alkyl halide, a homo-bifunctional pyridyl disulfide, a homo-bifunctional aryl halide, a homo-bifunctional hydrazide, a homo-bifunctional diazonium derivative and a homo-bifunctional photoreactive compound may be used. Also included are hetero-bifunctional compounds, for example, compounds having an amine-reactive and a sulfhydryl-reactive group, compounds with an amine-reactive and a photoreactive group and compounds with a carbonyl-reactive and a sulfhydryl-reactive group.

[0112] Specific examples of such homo-bifunctional cross-linking agents include the bifunctional N-hydroxysuccinimide esters dithiobis(succinimidylpropionate), disuccinimidyl suberate, and disuccinimidyl tartrate; the bifunctional imido-esters dimethyl adipimidate, dimethyl pimelimidate, and dimethyl suberimidate; the bifunctional sulfhydryl-reactive crosslinkers 1,4-di-[3'-(2'-pyridyldithio) propionamido]butane, bismaleimidohexane, and bis-N-maleimido-1, 8-octane; the bifunctional aryl halides 1,5-difluoro-2,4-dinitrobenzene and 4,4'-difluoro-3,3'-dinitrophenylsulfone; bifunctional photoreactive agents such as bis-[b-(4-azidosalicylamido)ethyl]disulfide; the bifunctional aldehydes formaldehyde, malondialdehyde, succinaldehyde, glutaraldehyde, and

adipaldehyde; a bifunctional epoxide such as 1,4-butaneodiol diglycidyl ether; the bifunctional hydrazides adipic acid dihydrazide, carbohydrazide, and succinic acid dihydrazide; the bifunctional diazoniums o-tolidine, diazotized and bis-diazotized benzidine; the bifunctional alkylhalides N1N'-ethylene-bis(iodoacetamide), N1N'-hexamethylene-bis(iodoacetamide), N1N'-undecamethylene-bis(iodoacetamide), as well as benzylhalides and halomustards, such as a1a'-diiodo-p-xylene sulfonic acid and tri(2-chloroethyl)amine, respectively.

[0113] Examples of common hetero-bifunctional cross-linking agents that may be used to effect the conjugation of proteins to peptides include, but are not limited to, SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate), MBS (m-maleimidobenzoyl-N-hydroxysuccinimide ester), SIAB (N-succinimidyl(4-iodoacteyl)aminobenzoate), SMPB (succinimidyl-4-(p-maleimidophenyl)butyrate), GMBS (N-(γ-maleimidobutyryloxy)succinimide ester), MPBH (4-(4-N-maleimidopohenyl) butyric acid hydrazide), M2C2H (4-(N-maleimidomethyl) cyclohexane-1-carboxyl-hydrazide), SMPT (succinimidyloxycarbonyl-α-methyl- α -(2-pyridyldithio)toluene), and SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate).

[0114] Cross-linking may be accomplished by coupling a carbonyl group to an amine group or to a hydrazide group by reductive amination.

[0115] Peptides of the disclosure also may be formulated as non-covalent attachment of monomers through ionic, adsorptive, or biospecific interactions. Complexes of peptides with highly positively or negatively charged molecules may be done through salt bridge formation under low ionic strength environments, such as in deionized water. Large complexes can be created using charged polymers such as poly-(L-glutamic acid) or poly-(L-lysine) which contain numerous negative and positive charges, respectively. Adsorption of peptides may be done to surfaces such as microparticle latex beads or to other hydrophobic polymers, forming non-covalently associated peptide-superantigen complexes effectively mimicking cross-linked or chemically polymerized protein. Finally, peptides may be non-covalently linked through the use of biospecific interactions between other molecules. For instance, utilization of the strong affinity of biotin for proteins such as avidin or streptavidin or their derivatives could be used to form peptide complexes. These biotin-binding proteins contain four binding sites that can interact with biotin in solution or be covalently attached to another molecule. (See Wilchek (1988)

Anal. Biochem. 171:1-32). Peptides can be modified to possess biotin groups using common biotinylation reagents such as the N-hydroxysuccinimidyl ester of D-biotin (NHS-biotin) which reacts with available amine groups on the protein. Biotinylated peptides then can be incubated with avidin or streptavidin to create large complexes. The molecular mass of such polymers can be regulated through careful control of the molar ratio of biotinylated peptide to avidin or streptavidin.

[0116] Also provided by this application are the peptides and polypeptides described herein conjugated to a label, e.g., a fluorescent or bioluminescent label, for use in the diagnostic methods. For example, detectably labeled peptides and polypeptides can be bound to a column and used for the detection and purification of antibodies. Suitable fluorescent labels include, but are not limited to, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue™, and Texas Red. Other suitable optical dyes are described in Haugland, Richard P. (1996) Molecular Probes Handbook.

[0117] The polypeptides of this disclosure also can be combined with various liquid phase carriers, such as sterile or aqueous solutions, pharmaceutically acceptable carriers, suspensions and emulsions. Examples of non-aqueous solvents include propyl ethylene glycol, polyethylene glycol and vegetable oils. When used to prepare antibodies, the carriers also can include an adjuvant that is useful to non-specifically augment a specific immune response. A skilled artisan can easily determine whether an adjuvant is required and select one. However, for the purpose of illustration only, suitable adjuvants include, but are not limited to, Freund's Complete Adjuvant, Freund's Incomplete Adjuvant and mineral salts.

Therapeutic Antibody Compositions

[0118] This disclosure also provides an antibody capable of modulating BMP signaling by forming a complex with a BMP protein, a protein or polypeptide in the BMP signaling pathway, or a protein or polypeptide, such as a BMP agonist or antagonist, that modulates BMP signaling. In some embodiments, the antibody is a modified polypeptide of the antibody as described herein. In some embodiments, the antibody is a blocking fragment of the antibody. These antibodies can target intracellular or extracellular signaling elements and therefore either promote or antagonize BMP function. The BMP signaling

pathway is described in Anderson et al. (2008) Nature Chem. Bio. 4(2):15-16. Antagonist include, for example noggin and/or chordin proteins.

[0119] The term "antibody" includes polyclonal antibodies and monoclonal antibodies, antibody fragments, as well as derivatives thereof (described above). The antibodies include, but are not limited to mouse, rat, and rabbit or human antibodies. Antibodies can be produced in cell culture, in phage, or in various animals, including but not limited to cows, rabbits, goats, mice, rats, hamsters, guinea pigs, sheep, dogs, cats, monkeys, chimpanzees, apes, *etc*. The antibodies are also useful to identify and purify therapeutic polypeptides.

[0120] This disclosure also provides an antibody-peptide complex comprising antibodies described above and a polypeptide that specifically binds to the antibody. In one aspect the polypeptide is the polypeptide against which the antibody was raised. In one aspect the antibody-peptide complex is an isolated complex. In a further aspect, the antibody of the complex is, but not limited to, a polyclonal antibody, a monoclonal antibody, a humanized antibody or an antibody derivative described herein. Either or both of the antibody or peptide of the antibody-peptide complex can be detectably labeled. In one aspect, the antibody-peptide complex of the disclosure can be used as a control or reference sample in diagnostic or screening assays.

[0121] Polyclonal antibodies of the disclosure can be generated using conventional techniques known in the art and are well-described in the literature. Several methodologies exist for production of polyclonal antibodies. For example, polyclonal antibodies are typically produced by immunization of a suitable mammal such as, but not limited to, chickens, goats, guinea pigs, hamsters, horses, mice, rats, and rabbits. An antigen is injected into the mammal, which induces the B-lymphocytes to produce IgG immunoglobulins specific for the antigen. This IgG is purified from the mammals serum. Variations of this methodology include modification of adjuvants, routes and site of administration, injection volumes per site and the number of sites per animal for optimal production and humane treatment of the animal. For example, adjuvants typically are used to improve or enhance an immune response to antigens. Most adjuvants provide for an injection site antigen depot, which allows for a slow release of antigen into draining lymph nodes. Other adjuvants include surfactants which promote concentration of protein antigen molecules over a large surface area and immunostimulatory molecules. Non-

limiting examples of adjuvants for polyclonal antibody generation include Freund's adjuvants, Ribi adjuvant system, and Titermax. Polyclonal antibodies can be generated using methods described in U.S. Patent Nos. 7,279,559; 7,119,179; 7,060,800; 6,709,659; 6,656,746; 6,322,788; 5,686,073; and 5,670,153.

[0122] The monoclonal antibodies of the disclosure can be generated using conventional hybridoma techniques known in the art and well-described in the literature. For example, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as, but not limited to, Sp2/0, Sp2/0-AG14, NSO, NS1, NS2, AE-1, L.5, >243, P3X63Ag8.653, Sp2 SA3, Sp2 MAI, Sp2 SS1, Sp2 SA5, U397, MLA 144, ACT IV, MOLT4, DA-1, JURKAT, WEHI, K-562, COS, RAJI, NIH 3T3, HL-60, MLA 144, NAMAIWA, NEURO 2A, CHO, PerC.6, YB2/O) or the like, or heteromyelomas, fusion products thereof, or any cell or fusion cell derived therefrom, or any other suitable cell line as known in the art (see, e.g., www.atcc.org, www.lifetech.com., last accessed on November 26, 2007, and the like), with antibody producing cells, such as, but not limited to, isolated or cloned spleen, peripheral blood, lymph, tonsil, or other immune or B cell containing cells, or any other cells expressing heavy or light chain constant or variable or framework or CDR sequences, either as endogenous or heterologous nucleic acid, as recombinant or endogenous, viral, bacterial, algal, prokaryotic, amphibian, insect, reptilian, fish, mammalian, rodent, equine, ovine, goat, sheep, primate, eukaryotic, genomic DNA, cDNA, rDNA, mitochondrial DNA or RNA, chloroplast DNA or RNA, hnRNA, mRNA, tRNA, single, double or triple stranded, hybridized, and the like or any combination thereof. Antibody producing cells can also be obtained from the peripheral blood or, preferably the spleen or lymph nodes, of humans or other suitable animals that have been immunized with the antigen of interest. Any other suitable host cell can also be used for expressing-heterologous or endogenous nucleic acid encoding an antibody, specified fragment or variant thereof, of the present disclosure. The fused cells (hybridomas) or recombinant cells can be isolated using selective culture conditions or other suitable known methods, and cloned by limiting dilution or cell sorting, or other known methods.

[0123] In one embodiment, the antibodies described herein can be generated using a Multiple Antigenic Peptide (MAP) system. The MAP system utilizes a peptidyl core of three or seven radially branched lysine residues, on to which the antigen peptides of

interest can be built using standard solid-phase chemistry. The lysine core yields the MAP bearing about 4 to 8 copies of the peptide epitope depending on the inner core that generally accounts for less than 10% of total molecular weight. The MAP system does not require a carrier protein for conjugation. The high molar ratio and dense packing of multiple copies of the antigenic epitope in a MAP has been shown to produce strong immunogenic response. This method is described in U.S. Patent No. 5,229,490 and is herein incorporated by reference in its entirety.

[0124] Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, but not limited to, methods that select recombinant antibody from a peptide or protein library (e.g., but not limited to, a bacteriophage, ribosome, oligonucleotide, RNA, cDNA, or the like, display library; e.g., as available from various commercial vendors such as Cambridge Antibody Technologies (Cambridgeshire, UK), MorphoSys (Martinsreid/Planegg, Del.), Biovation (Aberdeen, Scotland, UK) BioInvent (Lund, Sweden), using methods known in the art. See U.S. Patent Nos. 4,704,692; 5,723,323; 5,763,192; 5,814,476; 5,817,483; 5,824,514; 5,976,862. Alternative methods rely upon immunization of transgenic animals (e.g., SCID mice, Nguyen et al. (1977) Microbiol. Immunol. 41:901-907 (1997); Sandhu et al.(1996) Crit. Rev. Biotechnol. 16:95-118; Eren et al. (1998) Immunol. 93:154-161 that are capable of producing a repertoire of human antibodies, as known in the art and/or as described herein. Such techniques, include, but are not limited to, ribosome display (Hanes et al. (1997) Proc. Natl. Acad. Sci. USA, 94:4937-4942; Hanes et al.(1998) Proc. Natl. Acad. Sci. USA, 95:14130-14135); single cell antibody producing technologies (e.g., selected lymphocyte antibody method ("SLAM") (U.S. Patent No. 5,627,052, Wen et al. (1987) J. Immunol. 17:887-892; Babcook et al., Proc. Natl. Acad. Sci. USA (1996) 93:7843-7848); gel microdroplet and flow cytometry (Powell et al. (1990) Biotechnol. 8:333-337; One Cell Systems, (Cambridge, Mass).; Gray et al. (1995) J. Imm. Meth. 182:155-163; and Kenny et al. (1995) Bio. Technol. 13:787-790); B-cell selection (Steenbakkers et al. (1994) Molec. Biol. Reports 19:125-134.

[0125] Antibody derivatives of the present disclosure can also be prepared by delivering a polynucleotide encoding an antibody of this disclosure to a suitable host such as to provide transgenic animals or mammals, such as goats, cows, horses, sheep, and the like, that produce such antibodies in their milk. These methods are known in the art and are

described for example in U.S. Patent Nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; and 5,304,489.

[0126] The term "antibody derivative" includes post-translational modification to linear polypeptide sequence of the antibody or fragment. For example, U.S. Patent No. 6,602,684 B1 describes a method for the generation of modified glycol-forms of antibodies, including whole antibody molecules, antibody fragments, or fusion proteins that include a region equivalent to the Fc region of an immunoglobulin, having enhanced Fc-mediated cellular toxicity, and glycoproteins so generated.

[0127] Antibody derivatives also can be prepared by delivering a polynucleotide of this disclosure to provide transgenic plants and cultured plant cells (e.g., but not limited to tobacco, maize, and duckweed) that produce such antibodies, specified portions or variants in the plant parts or in cells cultured therefrom. For example, Cramer et al. (1999) Curr. Top. Microbol. Immunol. 240:95-118 and references cited therein, describe the production of transgenic tobacco leaves expressing large amounts of recombinant proteins, e.g., using an inducible promoter. Transgenic maize have been used to express mammalian proteins at commercial production levels, with biological activities equivalent to those produced in other recombinant systems or purified from natural sources. See, e.g., Hood et al. (1999) Adv. Exp. Med. Biol. 464:127-147 and references cited therein. Antibody derivatives have also been produced in large amounts from transgenic plant seeds including antibody fragments, such as single chain antibodies (scFv's), including tobacco seeds and potato tubers. See, e.g., Conrad et al.(1998) Plant Mol. Biol. 38:101-109 and reference cited therein. Thus, antibodies of the present disclosure can also be produced using transgenic plants, according to know methods.

[0128] Antibody derivatives also can be produced, for example, by adding exogenous sequences to modify immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic. Generally part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the variable and constant regions are replaced with human or other amino acids.

[0129] In general, the CDR residues are directly and most substantially involved in influencing antigen binding. Humanization or engineering of antibodies of the present

disclosure can be performed using any known method such as, but not limited to, those described in U.S. Patent Nos. 5,723,323; 5,976,862; 5,824,514; 5,817,483; 5,814,476; 5,763,192; 5,723,323; 5,766,886; 5,714,352; 6,204,023; 6,180,370; 5,693,762; 5,530,101; 5,585,089; 5,225,539; and 4,816,567.

[0130] Techniques for making partially to fully human antibodies are known in the art and any such techniques can be used. According to one embodiment, fully human antibody sequences are made in a transgenic mouse which has been engineered to express human heavy and light chain antibody genes. Multiple strains of such transgenic mice have been made which can produce different classes of antibodies. B cells from transgenic mice which are producing a desirable antibody can be fused to make hybridoma cell lines for continuous production of the desired antibody. (See for example, Russel et al. (2000) Infection and Immunity April 2000:1820-1826; Gallo et al. (2000) European J. of Immun. 30:534-540; Green (1999) J. of Immun. Methods 231:11-23; Yang et al. (1999A) J. of Leukocyte Biology 66:401-410; Yang (1999B) Cancer Research 59(6):1236-1243; Jakobovits (1998) Advanced Drug Delivery Reviews 31:33-42; Green and Jakobovits (1998) J. Exp. Med. 188(3):483-495; Jakobovits (1998) Exp. Opin. Invest. Drugs 7(4):607-614; Tsuda et al. (1997) Genomics 42:413-421; Sherman-Gold (1997) Genetic Engineering News 17(14); Mendez et al. (1997) Nature Genetics 15:146-156; Jakobovits (1996) Weir's Handbook of Experimental Immunology, The Integrated Immune System Vol. IV, 194.1-194.7; Jakobovits (1995) Current Opinion in Biotechnology 6:561-566; Mendez et al. (1995) Genomics 26:294-307; Jakobovits (1994) Current Biology 4(8):761-763; Arbones et al. (1994) Immunity 1(4):247-260; Jakobovits (1993) Nature 362(6417):255-258; Jakobovits et al. (1993) Proc. Natl. Acad. Sci. USA 90(6):2551-2555; and U.S. Patent No. 6,075,181.)

[0131] The antibodies of this disclosure also can be modified to create chimeric antibodies. Chimeric antibodies are those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species. See, e.g., U.S. Patent No. 4,816,567.

[0132] Alternatively, the antibodies of this disclosure can also be modified to create veneered antibodies. Veneered antibodies are those in which the exterior amino acid residues of the antibody of one species are judiciously replaced or "veneered" with those of a second species so that the antibodies of the first species will not be immunogenic in

the second species thereby reducing the immunogenicity of the antibody. Since the antigenicity of a protein is primarily dependent on the nature of its surface, the immunogenicity of an antibody could be reduced by replacing the exposed residues which differ from those usually found in another mammalian species antibodies. This judicious replacement of exterior residues should have little, or no, effect on the interior domains, or on the interdomain contacts. Thus, ligand binding properties should be unaffected as a consequence of alterations which are limited to the variable region framework residues. The process is referred to as "veneering" since only the outer surface or skin of the antibody is altered, the supporting residues remain undisturbed.

[0133] The procedure for "veneering" makes use of the available sequence data for human antibody variable domains compiled by Kabat et al. (1987) Sequences of Proteins of Immunological Interest, 4th ed., Bethesda, Md., National Institutes of Health, updates to this database, and other accessible U.S. and foreign databases (both nucleic acid and protein). Non-limiting examples of the methods used to generate veneered antibodies include EP 519596; U.S. Patent No. 6,797,492; and described in Padlan et al. (1991) Mol. Immunol. 28(4-5):489-498.

[0134] The term “antibody derivative” also includes “diabodies” which are small antibody fragments with two antigen-binding sites, wherein fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain. (See for example, EP 404,097; WO 93/11161; and Hollinger et al., (1993) Proc. Natl. Acad. Sci. USA 90:6444-6448.) By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. (See also, U.S. Patent No. 6,632,926 to Chen et al. which discloses antibody variants that have one or more amino acids inserted into a hypervariable region of the parent antibody and a binding affinity for a target antigen which is at least about two fold stronger than the binding affinity of the parent antibody for the antigen.)

[0135] The term “antibody derivative” further includes “linear antibodies”. The procedure for making linear antibodies is known in the art and described in Zapata et al. (1995) Protein Eng. 8(10):1057-1062. Briefly, these antibodies comprise a pair of tandem Fd segments (V_H -C_H 1-VH -C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[0136] The antibodies of this disclosure can be recovered and purified from recombinant cell cultures by known methods including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography ("HPLC") can also be used for purification.

[0137] Antibodies of the present disclosure include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a eukaryotic host, including, for example, yeast, higher plant, insect and mammalian cells, or alternatively from a prokaryotic cells as described above.

[0138] If a monoclonal antibody being tested binds with protein or polypeptide, then the antibody being tested and the antibodies provided by the hybridomas of this disclosure are equivalent. It also is possible to determine without undue experimentation, whether an antibody has the same specificity as the monoclonal antibody of this disclosure by determining whether the antibody being tested prevents a monoclonal antibody of this disclosure from binding the protein or polypeptide with which the monoclonal antibody is normally reactive. If the antibody being tested competes with the monoclonal antibody of the disclosure as shown by a decrease in binding by the monoclonal antibody of this disclosure, then it is likely that the two antibodies bind to the same or a closely related epitope. Alternatively, one can pre-incubate the monoclonal antibody of this disclosure with a protein with which it is normally reactive, and determine if the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of this disclosure.

[0139] The term "antibody" also is intended to include antibodies of all isotypes. Particular isotypes of a monoclonal antibody can be prepared either directly by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class switch variants using the procedure described in Steplewski et al. (1985) Proc. Natl. Acad. Sci. USA 82:8653 or Spira et al. (1984*)* J. Immunol. Methods 74:307.

[0140] The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the disclosure can also be accomplished by one of ordinary skill in the art by producing anti-idiotypic antibodies. Herlyn et al. (1986) Science 232:100. An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the monoclonal antibody produced by the hybridoma of interest.

[0141] Idiotypic identity between monoclonal antibodies of two hybridomas demonstrates that the two monoclonal antibodies are the same with respect to their recognition of the same epitopic determinant. Thus, by using antibodies to the epitopic determinants on a monoclonal antibody it is possible to identify other hybridomas expressing monoclonal antibodies of the same epitopic specificity.

[0142] It is also possible to use the anti-idiotype technology to produce monoclonal antibodies which mimic an epitope. For example, an anti-idiotypic monoclonal antibody made to a first monoclonal antibody will have a binding domain in the hypervariable region which is the mirror image of the epitope bound by the first monoclonal antibody. Thus, in this instance, the anti-idiotypic monoclonal antibody could be used for immunization for production of these antibodies.

[0143] In some aspects of this disclosure, it will be useful to detectably or therapeutically label the antibody. Suitable labels are described *supra*. Methods for conjugating antibodies to these agents are known in the art. For the purpose of illustration only, antibodies can be labeled with a detectable moiety such as a radioactive atom, a chromophore, a fluorophore, or the like. Such labeled antibodies can be used for diagnostic techniques, either *in vivo*, or in an isolated test sample.

[0144] The coupling of antibodies to low molecular weight haptens can increase the sensitivity of the antibody in an assay. The haptens can then be specifically detected by means of a second reaction. For example, it is common to use haptens such as biotin, which reacts avidin, or dinitrophenol, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies. See, Harlow and Lane (1988) *supra*.

[0145] Antibodies can be labeled with a detectable moiety such as a radioactive atom, a chromophore, a fluorophore, or the like. Such labeled antibodies can be used for diagnostic techniques, either *in vivo*, or in an isolated test sample. Antibodies can also be conjugated, for example, to a pharmaceutical agent, such as chemotherapeutic drug or a

toxin. They can be linked to a cytokine, to a ligand, to another antibody. Suitable agents for coupling to antibodies to achieve an anti-tumor effect include cytokines, such as interleukin 2 (IL-2) and Tumor Necrosis Factor (TNF); photosensitizers, for use in photodynamic therapy, including aluminum (III) phthalocyanine tetrasulfonate, hematoporphyrin, and phthalocyanine; radionuclides, such as iodine-131 (^{131}I), yttrium-90 (^{90}Y), bismuth-212 (^{212}Bi), bismuth-213 (^{213}Bi), technetium-99m (^{99m}Tc), rhenium-186 (^{186}Re), and rhenium-188 (^{188}Re); antibiotics, such as doxorubicin, adriamycin, daunorubicin, methotrexate, daunomycin, neocarzinostatin, and carboplatin; bacterial, plant, and other toxins, such as diphtheria toxin, pseudomonas exotoxin A, staphylococcal enterotoxin A, abrin-A toxin, ricin A (deglycosylated ricin A and native ricin A), TGF-alpha toxin, cytotoxin from Chinese cobra (naja naja atra), and gelonin (a plant toxin); ribosome inactivating proteins from plants, bacteria and fungi, such as restrictocin (a ribosome inactivating protein produced by *Aspergillus restrictus*), saporin (a ribosome inactivating protein from *Saponaria officinalis*), and RNase; tyrosine kinase inhibitors; ly207702 (a difluorinated purine nucleoside); liposomes containing anti cystic agents (*e.g.*, antisense oligonucleotides, plasmids which encode for toxins, methotrexate, *etc.*); and other antibodies or antibody fragments, such as F(ab).

[0146] The antibodies of the disclosure also can be bound to many different carriers. Thus, this disclosure also provides compositions containing the antibodies and another substance, active or inert. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the disclosure. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such, using routine experimentation.

Pharmaceutical Compositions

[0147] In one aspect, the disclosure provides compositions used in the methods. In some embodiments, the compositions are small molecules that enhance or inhibit BMP signaling. In some embodiments, the compositions are polynucleotides that encode BMP proteins, encode polypeptides enhancing or inhibiting BMP signaling, or enhance or inhibit expression of BMP proteins, or polypeptides enhancing or inhibiting BMP signaling. In some embodiments, the compositions are isolated or recombinant BMP

proteins, or isolated or recombinant polypeptides enhancing or inhibiting BMP signaling. Examples of each of these agents are described in this application and are the active agents in the pharmaceutical compositions.

[0148] In some aspect, the composition further comprises a pharmaceutically acceptable carrier, e.g., DMSO. In another aspect, the compositions contain carriers that modulate (controlled release) the release of the active agent when administered to a subject in need thereof. For example, the carriers can also include transdermal

[0149] The pharmaceutical compositions of the disclosure can be manufactured by methods well known in the art such as conventional granulating, mixing, dissolving, encapsulating, lyophilizing, or emulsifying processes, among others. Compositions may be produced in various forms, including granules, precipitates, or particulates, powders, including freeze dried, rotary dried or spray dried powders, amorphous powders, injections, emulsions, elixirs, suspensions or solutions. Formulations may optionally contain stabilizers, pH modifiers, surfactants, bioavailability modifiers and combinations of these.

[0150] Pharmaceutical formulations may be prepared as liquid suspensions or solutions using a sterile liquid, such as oil, water, alcohol, and combinations thereof. Pharmaceutically suitable surfactants, suspending agents or emulsifying agents, may be added for oral or parenteral administration. Suspensions may include oils, such as peanut oil, sesame oil, cottonseed oil, corn oil and olive oil. Suspension preparation may also contain esters of fatty acids, such as ethyl oleate, isopropyl myristate, fatty acid glycerides and acetylated fatty acid glycerides. Suspension formulations may include alcohols, such as ethanol, isopropyl alcohol, hexadecyl alcohol, glycerol and propylene glycol. Ethers, such as poly(ethyleneglycol), petroleum hydrocarbons, such as mineral oil and petrolatum, and water may also be used in suspension formulations.

[0151] The compositions of this disclosure are formulated for pharmaceutical administration to a mammal, preferably a human being. Such pharmaceutical compositions of the disclosure may be administered in a variety of ways, preferably topically or intradermally.

[0152] Pharmaceutically acceptable excipients and carriers and dosage forms are generally known to those skilled in the art and are included in the disclosure. It should be

understood that a specific dosage and treatment regimen for any particular patient will depend upon a variety of factors, including the activity of the specific antidote employed, the age, body weight, general health, sex and diet, renal and hepatic function of the patient, and the time of administration, rate of excretion, drug combination, judgment of the treating physician or veterinarian and severity of the particular disease being treated.

[0153] For prophylactic administration, the compound can be administered to a patient at risk of developing one of the previously described conditions. For example, prophylactic administration can be applied to avoid the onset of symptoms in a patient diagnosed with the underlying disorder such as alopecia or a genetic predisposition to alocpecia.

[0154] The amount of compound administered will depend upon a variety of factors, including, for example, the particular indication being treated, the mode of administration, whether the desired benefit is prophylactic or therapeutic, the severity of the indication being treated and the age and weight of the patient, and the bioavailability of the particular active compound. Determination of an effective dosage is well within the capabilities of those skilled in the art.

[0155] Effective dosages can be estimated initially from *in vitro* assays. For example, an initial dosage for use in animals can be formulated to achieve a local (topical) or circulating blood or serum concentration of active compound that is at or above an IC_{50} of the particular compound as measured in as *in vitro* assay. Calculating dosages to achieve such circulating blood or serum concentrations taking into account the bioavailability of the particular compound is well within the capabilities of skilled artisans. For guidance, the reader is referred to Fingl & Woodbury, "General Principles," In: Goodman and Gilman's The Pharmaceutical Basis of Therapeutics, Chapter 1, pp. 1-46, latest edition, Pergamagon Press, and the references cited therein.

[0156] Initial dosages can also be estimated from *in vivo* data, such as animal models. Animal models useful for testing the efficacy of compounds to treat or prevent the various diseases described above are well-known in the art. Ordinarily skilled artisans can routinely adapt such information to determine dosages suitable for human administration.

[0157] Dosage amounts will typically be in the range of from about 0.0001 or 0.001 or 0.01 mg/kg/day to about 100 mg/kg/day, but can be higher or lower, depending upon,

among other factors, the activity of the compound, its bioavailability, the mode of administration, and various factors discussed above. Dosage amount and interval can be adjusted individually to provide plasma levels of the compound(s) which are sufficient to maintain therapeutic or prophylactic effect. For example, the compounds can be administered once per week, several times per week (*e.g.*, every other day), once per day, or multiple times per day, depending upon, among other things, the mode of administration, the specific indication being treated, and the judgment of the prescribing physician. In cases of local administration or selective uptake, such as local topical administration, the effective local concentration of active compound(s) may not be related to plasma concentration. Skilled artisans will be able to optimize effective local dosages without undue experimentation.

[0158] Preferably, the compound(s) will provide therapeutic or prophylactic benefit without causing substantial toxicity. Toxicity of the compound(s) can be determined using standard pharmaceutical procedures. The dose ratio between toxic and therapeutic (or prophylactic) effect is the therapeutic index. Compounds(s) that exhibit high therapeutic indices are preferred.

[0159] One aspect of the disclosure comprises small molecules that enhance or inhibit BMP signaling. Small molecule agonist and antagonists for other signaling pathways exist. Interaction of these small molecules with pathways including, but not limited to, WNT, SHH and FGF will also have direct or indirect impact on BMP signaling thus serve as effective modulator of hair growth via methods disclosed in this disclosure. Non-limiting examples include the proteins noggin, chordin, and dorsomorphin, a small molecule inhibitor of BMP signaling. For more details of the mechanism and composition of dorsomorphin, see Hao et al. (2008) PLoS ONE, 3(8):e2904 and Yu et al. (2008) Nat Chem Biol. 4(1):33-41. Dorsomorphin was reported to selectively inhibit the BMP receptors, type I: ALK2, ALK3 and ALK6 and thus "blocks BMP-mediated SMAD1/5/8 phosphorylation". Dorsomorphin has preferential specificity toward inhibiting BMP versus TGF-beta and activin signaling. In published reports, Dorsomorphin is characterized by low toxicity. Dorsomorphin is currently commercially available from several vendors. Dorsomorphin can be delivered into skin to lower macro-environmental BMP signaling and create favorable conditions for hair growth to occur. A unique property of Dorsomorphin is that it is a small molecule and is soluble in DMSO.

DMSO is known to significantly facilitate trans-dermal delivery of small molecule drugs. This enhancing effect of DMSO on skin penetration can be used in non-invasive method of pharmacological modulation of dermal macro-environment. Treatment procedure thus consists of simply applying liquid form of Dorsomorphin in DMSO onto the surface of intact skin. Dorsomorphin in DMSO can be made in form of cream. Cream can be simply rubbed onto intact skin. Additional agents can be co-formulated or delivered concomitantly or sequentially with the above noted agents, e.g., minoxidil. The formulations can be for immediate or controlled release of the active ingredients.

[0160] Another aspect of the disclosure comprises polynucleotides that encode BMP proteins, encode polypeptides enhancing or inhibiting BMP signaling, or enhance or inhibit expression of BMP proteins, or polypeptides enhancing or inhibiting BMP signaling. Examples of such polynucleotides include, but are not limited to, nucleotides encoding BMP proteins, ligands to BMP proteins and proteins in the BMP signal pathway and polypeptides homologous or having at least 80 %, or alternatively, at least 85 %, or alternatively at least 90 %, or alternatively at least 95 %, or alternatively at least 98% seqeuence identity to these proteins. Non-limiting examples also include siRNA that interferences with expression of such polypeptides. Additional agents can be co-formulated or delivered concomitantly or sequentially with the above noted agents, e.g., minoxidil. The formulations can be for immediate or controlled release of the active ingredients.

[0161] Another aspect of the disclosure comprises isolated or recombinant BMP proteins, or isolated or recombinant polypeptides enhancing or inhibiting BMP signaling. In some aspect, the composition further comprises a pharmaceutically acceptable carrier. The polypeptides and polypeptide complexes of the disclosure can be used in a variety of formulations, which may vary depending on the intended use. For example, one or more can be covalently or non-covalently linked (complexed) to various other molecules, the nature of which may vary depending on the particular purpose. For example, a peptide of the disclosure can be covalently or non-covalently complexed to a macromolecular carrier, including, but not limited to, natural and synthetic polymers, proteins, polysaccharides, polypeptides (amino acids), polyvinyl alcohol, polyvinyl pyrrolidone, and lipids. A peptide can be conjugated to a fatty acid, for introduction into a liposome, see U.S. Patent No. 5,837,249. A peptide of the disclosure can be complexed covalently

or non-covalently with a solid support, a variety of which are known in the art and described herein. An antigenic peptide epitope of the disclosure can be associated with an antigen-presenting matrix such as an MHC complex with or without co-stimulatory molecules. Examples of protein carriers include, but are not limited to, superantigens, serum albumin, tetanus toxoid, ovalbumin, thyroglobulin, myoglobulin, and immunoglobulin.

[0162] Polypeptides may also be formulated as non-covalent attachment of monomers through ionic, adsorptive, or biospecific interactions. Complexes of peptides with highly positively or negatively charged molecules may be done through salt bridge formation under low ionic strength environments, such as in deionized water. Large complexes can be created using charged polymers such as poly-(L-glutamic acid) or poly-(L-lysine) which contain numerous negative and positive charges, respectively. Adsorption of peptides may be done to surfaces such as microparticle latex beads or to other hydrophobic polymers, forming non-covalently associated peptide-superantigen complexes effectively mimicking cross-linked or chemically polymerized protein. Finally, peptides may be non-covalently linked through the use of biospecific interactions between other molecules. For instance, utilization of the strong affinity of biotin for proteins such as avidin or streptavidin or their derivatives could be used to form peptide complexes. These biotin-binding proteins contain four binding sites that can interact with biotin in solution or be covalently attached to another molecule. (See Wilchek (1988) Anal. Biochem. 171:1-32). Peptides can be modified to possess biotin groups using common biotinylation reagents such as the N-hydroxysuccinimidyl ester of D-biotin (NHS-biotin) which reacts with available amine groups on the protein.

[0163] The polypeptides also can be combined with various liquid phase carriers, such as sterile or aqueous solutions, pharmaceutically acceptable carriers, suspensions and emulsions for immediate or controlled release. Examples of non-aqueous solvents include propyl ethylene glycol, polyethylene glycol and vegetable oils. When used to prepare antibodies, the carriers also can include an adjuvant that is useful to non-specifically augment a specific immune response. A skilled artisan can easily determine whether an adjuvant is required and select one. However, for the purpose of illustration only, suitable adjuvants include, but are not limited to, Freund's Complete Adjuvant, Freund's Incomplete Adjuvant and mineral salts.

Kits

[0164] An aspect of the disclosure provides a kit for inhibiting hair growth in a tissue having a hair follicle, comprising an effective amount of an agent that augments BMP in a pharmaceutically acceptable carrier and instructions for use in inhibiting hair growth. Another aspect of the disclosure provides a kit for augmenting or promoting hair growth comprising an effective amount of an agent that inhibits BMP in a pharmaceutically acceptable carrier and instructions for use in augmenting or promoting hair growth. Additional agents can be co-formulated or delivered concomitantly or sequentially with the above noted agents, e.g., minoxidil and provided in the kits. The formulations can be for immediate or controlled release of the active ingredients.

[0165] In some embodiments, the pharmaceutically acceptable carrier in the kits is suitable for topical administration of the agent. Additional agents can be co-formulated or delivered concomitantly or sequentially with the above noted agents, e.g., minoxidil. The formulations can be for immediate or controlled release of the active ingredients.

[0166] In some embodiments, the pharmaceutically acceptable carrier further comprises a penetration or permeation enhancer.

[0167] Also provided are kits for administration of the compounds for treatment of disorders as described herein. Kits may further comprise suitable packaging and/or instructions for use of the compound. Kits may also comprise a means for the delivery of the at least one agonist or antagonist and instructions for administration. Alternatively, the kit provides the compound and reagents to prepare a composition for administration. The composition can be in a dry or lyophilized form or in a solution, particularly a sterile solution. When the composition is in a dry form, the reagent may comprise a pharmaceutically acceptable diluent for preparing a liquid formulation. The kit may contain a device for administration or for dispensing the compositions, including, but not limited to, syringe, pipette, transdermal patch, or inhalant.

[0168] The kits may include other therapeutic compounds for use in conjunction with the compounds described herein. These compounds can be provided in a separate form or mixed with the compounds of the present disclosure.

[0169] The kits will include appropriate instructions for preparation and administration of the composition, side effects of the compositions, and any other relevant information.

The instructions can be in any suitable format, including, but not limited to, printed matter, videotape, computer readable disk, or optical disc.

[0170] In another aspect of the disclosure, kits for treating an individual who suffers from or is susceptible to the conditions described herein are provided, comprising a container comprising a dosage amount of a composition, as disclosed herein, and instructions for use. The container can be any of those known in the art and appropriate for storage and delivery of oral, intravenous, topical, rectal, urethral, or inhaled formulations.

[0171] Kits may also be provided that contain sufficient dosages of the effective composition or compound to provide effective treatment for an individual for an extended period, such as a week, 2 weeks, 3, weeks, 4 weeks, 6 weeks, or 8 weeks or more.

Therapeutic, Diagnostic and Screening Utilities

[0172] This disclosure provides the follow therapeutic, diagnostic and screening utilities. In one aspect, the disclosure provides methods for facilitating hair growth in a tissue containing a hair follicle comprising administering to the tissue two or more agents selected from the group (a) a Bone Morphogenic Protein (BMP) signaling inhibitor, (b) a secreted frizzled-related protein 4 (Sfrp4) or dickkopf homolog 1 (Dkk1) inhibitor, (c) an NF-κB agonist, (d) a TNF-alpha, IL-1alpha or IL-1beta or an equivalent thereof, or (e) Follistatin, thereby facilitating hair growth.

[0173] Also provided are methods for treating alopecia in a subject having tissue containing a hair follicle, comprising administering to the tissue two or more agents selected from the group (a) a Bone Morphogenic Protein (BMP) signaling inhibitor, (b) a secreted frizzled-related protein 4 (Sfrp4) or dickkopf homolog 1 (Dkk1) inhibitor, (c) an NF-κB agonist, (d) a TNF-alpha, IL-1alpha or IL-1beta or an equivalent thereof, or (e) Follistatin, thereby treating alopecia in the subject.

[0174] In either of the above embodiments, the two or more agents can be any combination, including but not limited to (a), (b) and (e), (c) and (d) or (a) and (c).

[0175] In the above methods, the BMP inhibitor is one or more selected from the group of BMP antagonists including noggin, chordin, gremlin, sclerostin, follistatin, a small interference RNA (siRNA) or double strand RNA (dsRNA) that inhibits one or more

genes selected from the group consisting of BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 and BMP15, or an antibody or modified antibody that inhibits a BMP antagonist or activates or stabilizes a BMP protein selected from the group consisting of BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 and BMP15.

[0176] In a further aspect, the methods further comprise administering an effective amount of one or more of monoxidal, finasteride, spironolactone or a second agent enhancing hair growth.

[0177] In each of the above methods, the method may further comprise ablating the tissue prior to administration of the agent and/or further comprises administration of penetration enhancer prior to or concomitantly with administration of the agent.

[0178] In each of the above methods, administration of the agents may be by any one or more of topical or interdermally, using creams, gels, solutions, sprays, microneedles or ionotophoresis or the like.

[0179] Compositions described above, such as small molecules BMP agonists or antagonists, polynucleotides and polypeptides both agonistic and antagonistic, can be administered to the subject in need of. In one aspect, the composition is directly delivered into or onto the skin. In another aspect, the composition is delivered during telogen phase or during competent telogen phase of the hair follicle which can be determined by one skilled in the art and briefly described herein. In another aspect, delivery can be made via microneedles. Microneedles allow penetrating stratum corneum – the outer layer of epidermis, responsible for the most of skin's barrier properties. Since microneedles do not reach into deeper skin layer, they do not cause painful sensations.

[0180] BMP proteins have been successfully delivered intracutaneously via single glass microneedles. Delivery of BMP proteins during competent telogen phase rendered treated skin refractory and prevented hair regeneration. For more standardized and simplified intracutaneous delivery hollow microneedle arrays can be used. Microneedle arrays contain hundreds of small individual microneedles evenly spaced apart on a platform. Microneedle array can also be connected to protein reservoir and injection mechanism. Such delivery apparatus can be realized in form of disposable injection syringe. Alternative delivery platform can be based on principle of micro-fluidics.

Microneedle / micro-fluidics device will provide slow intradermal delivery of compound at a constant rate over prolonged period of time. Such delivery platform can be realized in form of skin patch that can be attached over treatment area and worn without inconvenience for the patient.

[0181] Microneedles are commonly produced as multineedle arrays from silicon, metal, glass via means of micro-etching. Microneedles are designed to be 100 to 1000 mkm in length. When applied to the skin, micro-needle arrays puncture through stratum corneum into deeper layers of epidermis, while not penetrating all the way into the dermis. Thus, they effectively disrupt stratum corneum barrier, and yet at the same time to not reach cutaneous nerve endings or the capillaries, preventing pain, bleeding skin infection.

[0182] Micro-needles can be solid or hollow. If solid micro-needles are used, drug is applied to the skin in the form of spray, or gel upon removal of the micro-needle array. Use of hollow needles will allow direct passive drug delivery via produced micro-conduits. The active agent can be dry coated onto the inner surface of the micro-needles. It can also be co-administered as solution, suspension, emulsion or gel. Furthermore, use of hollow micro-needle arrays enable active drug delivery via combination of micro-needle array with microfluidic devices. These methods of stratum corneum disruption allow effective delivery of large molecular weight compounds such as peptides, proteins, and DNA constructs.

[0183] Microneedle arrays can be combined with syringe-like injection device to achieve simple protein delivery. Such delivery system can be realized in form of dermal patch, similar to ionophoretic insulin dermal patch.

[0184] Expression vectors, such as those expressing BMP ligands or antagonists, or naked cDNA for these genes can be delivered into skin using established intracutaneous gene delivery techniques, such as technique of electorporation or with the help of "gene gun". In order to express the proteins described herein, delivery of nucleic acid sequences encoding the gene of interest can be delivered by several techniques as described herein. A polynucleotide can be delivered to a cell or tissue using a gene delivery vehicle. Gene delivery vehicles may also include DNA/liposome complexes, micelles and targeted viral protein-DNA complexes. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this disclosure. To enhance delivery to a cell, the

nucleic acid or proteins of this disclosure can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens. Cell surface antigens characteristic to epidermis or hair follicle specific cell types should be used. Alternatively, antigens characteristic to stem cells should be used to target gene delivery into stem cells (such as hair follicle stem cells). In addition to the delivery of polynucleotides to a cell or cell population, direct introduction of the proteins described herein to the cell or cell population can be done by the non-limiting technique of protein transfection, alternatively culturing conditions that can enhance the expression and/or promote the activity of the proteins of this disclosure are other non-limiting techniques.

[0185] In one aspect, the composition for use in the methods further comprises a penetration enhancer or a carrier suitable for controlled release. Examples of penetration enhancers include, for example, propylene glycol/lauric acid, linalool, alpha terpineaol, carvacrol, limonene, menthone, eugenol, phloretin, polyphenol. The compositions can be formulated for delivery by spraying, topical administration, in a hydrogel or a transdermal patch.

[0186] In some embodiments, compositions of the disclosure can be delivered into the skin by injection with a carrier for long term release and effect. In one aspect, beads are used as a protein reservoir. In another aspect, the composition further comprises a biocompatible and/or dissolvable carrier. Non-limiting examples of biocompatible and/or dissolvable carriers include injectable collagen matrix, dissolvable hydrogel and injectable biocompatible and dissolvable polymers.

[0187] In another aspect, the composition or compositions can be co-administrated, or administered prior to or after administration of a second agent that enhances or inhibit hair growth. In one aspect, the second agent is minoxidil, a treatment for alopecia, commercially available as Rogaine or Regaine. In some embodiments, a combination of slow release excipients having two different rates of release where the composition of the disclosure is released over the course of a few hours, a day or more, followed by several days of release of the second agent. In another aspect, time release encapsulation comprising the compositions of the disclosure can be included in shampoo for convenient administration.

[0188] One aspect of the disclosure provides a method to determine if a test agent will likely modulate hair growth in a tissue having a hair follicle, comprising: (a) administering to a first tissue sample an amount of the test agent; (b) administering to a second tissue sample an effective amount of soluble BMP and/or (c) administering to a third tissue sample an effective amount of BMP antagonist, such as noggin; and (d) comparing the growth of hair in the first tissue sample to the growth in the second tissue sample and/or third tissue sample, wherein the test agent will likely modulate hair growth if the growth of hair in the first tissue sample is similar to the second tissue sample and/or third sample. In some embodiments, the method further comprises laser ablating or tape stripping of the tissue prior to administration of the agents. In yet some other aspects, the method further comprises administration of penetration enhancer prior to or concomitantly with administration of the agents.

[0189] The disclosure in one aspect provides a method to determine if a test agent will likely facilitate hair growth in a tissue of an animal, which animal comprises an expression cassette stably integrated into the animal genome, which expression cassette comprises a polynucleotide encoding a BMP protein under control of a skin-specific promoter, and then administering to the tissue an effective amount of the test agent, wherein formation of new hair or an increase of hair growth indicates that the test agent will likely facilitate hair growth. In some embodiments, the BMP protein is selected from the group consisting of BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 and BMP15.

[0190] The disclosure in another aspect provides a method to determine if a test agent will likely inhibit hair growth in a tissue of an animal, which animal comprises an expression cassette stably integrated into the animal genome, which expression cassette comprises a polynucleotide encoding a BMP antagonist under control of a skin-specific promoter, comprising administering to the tissue an effective amount of the test agent, wherein formation of new hair or an increase of hair growth indicates that the test agent will likely inhibit hair growth. In some embodiments, the BMP antagonist is selected from the group consisting of dorsomorphin, noggin, chordin, gremlin, sclerostin and follistatin. In one particular aspect, the BMP antagonist is noggin. In a further aspect the method is preformed in combination with additional agonist or antagonists as described above. The additional agents can be co-administered or delivered prior to or after the

other agent. In a yet further aspect, positive and negative controls are added. In a further aspect, the method is performed during the telogen phase of the hair follicles.

[0191] One aspect of the disclosure provides a method for determining if a subject having a condition is suitable for a treatment targeting BMP signaling, which condition comprises alopecia, which treatment comprises administration of an agent inhibiting BMP signaling, wherein an expression level of BMP mRNA or protein lower than a predetermined value indicates that the subject is suitable for the treatment. In a particular aspect, the BMP gene is BMP2. In another aspect, the BMP gene is BMP4.

[0192] In some embodiments, the predetermined value for evaluating BMP protein or mRNA expression level is determined in a subject, by comparing the areas of skin having high or low hair growth. A value that best separates expression values into a high hair growth group and a low hair growth group is the predetermined value. In some other embodiments, the predetermined value for evaluating BMP protein or mRNA expression level is determined in a group of subjects, by comparing the subjects with high hair growth to subjects with low hair growth. A value that best separates expression values into a high hair growth group and a low hair growth group is the predetermined cutoff value. In some embodiments, the subject is human.

[0193] mRNA expression values of a BMP gene may be determined with technology well known in the art. Examples of such technologies, without limitation, include real time PCR, in situ hybridization and microarray. In one aspect, the technology is real time PCR. Non-limiting examples of primers and probes to be used in real time PCR for human BMP genes include primer/probe sets commercially available from Applied Biosystems (Foster City, California): BMP1 (Assay ID: Hs00241807_m1), BMP2 (Assay ID: Hs00154192_m1), BMP3 (Assay ID: Hs00609639_m1), BMP4 (Assay ID: Hs00370078_m1), BMP5 (Assay ID: Hs00951007_m1), BMP6 (Assay ID: Hs00233470_m1), BMP7 (Assay ID: Hs00233476_m1), BMP8a (Assay ID: Hs00426893_g1), BMP8b (Assay ID: Hs01629120_s1), BMP10 (Assay ID: Hs00205566_m1) and BMP15 (Assay ID: Hs00193764_m1).

[0194] The agents and compositions of the present disclosure in all aspects as described above can be used in the manufacture of medicaments and for the treatment of humans

and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

Screening Compositions and Methods

[0195] The present disclosure also shows that, by combining predictive mathematical modeling with in vivo studies in mice and rabbits, a follicle progresses through cycling stages by continuous integration of inputs from intrinsic follicular and extrinsic environmental signals based on universal patterning principles.

[0196] This demonstrates that cell populations in which each cell cycles through stages in response to inputs from other cells in the population and environmental signals are useful in studying the communication between the cells and between the cells and the environment, in particular when the cycling can be easily monitored. Such a cell population, further, can be useful in elucidating cellular mechanisms associated with the communication and useful for drug screening. Methods of using such a cell population to carry out mechanistic studies or drug screen can be, for example, a cellular automata (CA) model, as illustrated in Example 2.

[0197] A non-limiting example of such a cell population is a mammalian skin containing hair follicles. Example 2 illustrates a large-scale hair regenerative system used to study regeneration of thousands of hairs at once on the skin surface. This hair regenerative system was used to find out principles that guide communication between stem cells during organ-wide (skin-wide) regeneration. There, cyclically appearing and disappearing hair pigmentation was used as the visual read-out of the regenerative behavior.

[0198] Using the hair regenerative system of Example 2, the applicants identified that the hair regenerative system followed principles of generic "Cellular Automata" (CA) model. It is thus contemplated that cells in other organs, in particular stem cells, as well as cells prepared in vitro, follow these principles as well.

[0199] Accordingly, one aspect of the present disclosure provides a cell population having a plurality of cells substantially evenly placed on a scaffold. For instance, the cells can be artificially pre-pattered onto the surface of the culture dish, similar to how hair stem cells are naturally patterned over the skin surface.

[0200] Methods of placing cells evenly on a scaffold are known in the art, e.g., with "cell printers", commercially available from Digilab, Inc. (Holliston, MA). In such an application, computer programs can be used to design the patterns of cells to be printed. Alternatively, preprint adhesive molecules (such as fibronectin) can be applied onto the surface of the scaffold which will then guide placement of the cells. Seeded cells would adhere well to fibronectin and won't adhere to hydrophobic plastic materials. This way cells will self-organize into patterns following distribution of adhesive molecules.

[0201] In another aspect, one or more cells of the cell population comprise a label indicative of cellular activities in the cells. In one aspect, the label is part of a gene reporter system, such as luciferase or fluorescent protein (RFP, GFP). An example is a fluorescent reporter of Wnt signaling pathway. Many of such reporters for key signaling pathways are currently available. With such a label, expression or activity changes of certain cellular components can be monitored visually or with appropriate instruments. For instance, fluorescent activity of all pre-patterned cells can be recorded with the camera and fluorescent patterns of their activity can be automatically analyzed with the software and compared to the predictions of the computer CA model.

[0202] In one aspect, an agent can be added into the cell population. If the agent alters cellular activity in the cells, changing patterns of fluorescent activity can be analyzed to match CA model predictions for potential system activators or inhibitors.

[0203] Thus, another aspect of the present disclosure provides a method of analyzing cell-cell communication and/or cell-environment interaction by analyzing signals emitted from a cell population of any of the above embodiments with an appropriate mathematical model. In one aspect, the model is a cellular automata model.

[0204] In another aspect, a method is provided for identifying the activity of an agent in a cell population by analyzing signals emitted from the cell population in contact with the agent with an appropriate mathematical model. In one aspect, the model is a cellular automata model.

[0205] The following examples are provide to illustrate select embodiments of the disclosure as disclosed and claimed herein.

EXPERIMENTAL EXAMPLES

Example 1. Quantitative hair plucking reveals stem cell regeneration is modulated by interactions with extra-follicular macro-environment

[0206] Stem cells in adult hair follicle can respond to injury signals and regenerate new hair filament. However, it is unknown how this regenerative behavior is regulated. Injury-induced activation of hair stem cells can occur via mechanism intrinsic to the follicle when injury occurs intra-follicular (plucking of a hair), or via extra-follicular mechanism, involving wound healing-induced cytokines. Here this example studied hair plucking-induced regeneration. Instead of large scale hair waxing this example performed careful quantitative plucking and accounted for the topological distribution of plucked hairs. It was found that, while the injury occurs within individual follicles, the regenerative response is affected by neighboring plucked follicles in a dose- and distance-dependent manner. Thus, the decision to regenerate is a decision made by a population of follicles rather than by each individual follicle. Mechanism of this collective regenerative response is dependent on activation of NF-κB through macro-environmental TNF-α signaling, which in turn activates intra-follicular canonical WNT pathway. It also showed that plucking-induced regeneration elicits secondary regenerative hair wave that propagates to the surrounding un-injured follicles via upregulated follistatin. Our results show the importance of quantitative topology in injury response and stem cell activation. The importance of macro-environmental modulators demonstrated here also opens new possibilities for treating alopecia and developing new organ regeneration strategies.

[0207] A majority of adult organs contain tissue-specific stem cells, which are typically kept quiescent, but can transiently become activated and participate in organ regeneration. In organs with high cellular turnaround, such as skin, gastro-intestinal (GI) tract and bone marrow, stem cell activities are controlled by complex physiological mechanisms, involving niche micro-environment, organ macro-environment and systemic components (Moore and Lemischka (2006) Science 311:1880-1885; Plikus et al. (2008) Nature 451:340-344; Méndez-Ferrer et al. (2008) Nature 452:442-447; Spiegel et al. (2008) Cell Stem Cell 3:484-492). Many aspects of such mechanisms have been already elucidated. In addition to physiological regeneration, most of the organs are capable of various degree of reparative regeneration in response to acute or chronic injuries. Mechanisms that control

stem cell activation upon injury and the degree of overlap between physiological and reparative activation mechanisms are not well understood.

[0208] Hair follicle is an excellent generic model for studying the regeneration of adult stem cells (Fuchs (2007) Nature 445:834-842). A hair follicle is a mini-organ composed of several epithelial and mesenchymal cell types. It contains a population of epithelial stem cells distinctly clustered into a bulge region (Cotsarelis et al. (1990) Cell 61:1329-1337). Activation of bulge stem cells marks initiation of a new hair regenerative cycle that consists of growth (anagen), regression (catagen) and resting (telogen) phases. Progression of the hair cycle and the appearance of new hairs can be used to effectively monitor stem cell activation events in individual hair follicles, as well as in large groups of follicles at once (Plikus et al. (2008) Nature 451:340-344). Activation of hair stem cells undergoes complex regulation by follicular micro-environment (primarily by dermal papilla) and most distinctly relies on WNT (Van Mater et al. (2003) Genes Dev. 15:1219-1224; Lowry et al. (2005) Genes Dev. 19:1596-1611; Enshell-Seijffers et al. (2010) Dev. Cell 18:633-642), BMP (Botchkarev et al. (2001) FASEB J. 15:2205-2214; Kobielak et al. (2007) PNAS 104:10063-10068) and FGF (Greco et al. (2009) Cell Stem Cell 4:155-169; Enshell-Seijffers et al. (2010) Dev Cell 18:633-642) signaling. In addition, larger dermal macro-environment modulates timing of hair regeneration and synchronizes it over the entire skin by co-opting BMP and WNT signaling cues from the micro-environment (Plikus et al. (2008) Nature 451:340-344). Hair follicles can also respond to systemic hormonal signals by initiating, halting or altering their regeneration such as during seasonal molting (Nixon et al. (1995) J Exp Zool 272:435-445). Thus, hair follicle is an open-end regenerative system, where stem cells activating signals can come both from within and from outside of the follicle.

[0209] Hair stem cells can also become activated upon injury, which can occur either outside of the follicle, such as upon skin wounding, or localize to the follicle itself, such as upon hair plucking. First is thought to be mediated by cytokines produced as part of the immune response by cells in wound macro-environment (Osaka et al. (2007) J Cell Biol 176:903-909; Jiang et al. (2010) J Dermatol Sci 60:143-150). Intra-follicular injury by hair plucking (Collins (1918) J Exp Zool 27:73-99; Silver and Chase (1970) Dev Biol 21:440-451) is thought to induce regeneration via an intra-follicular two-step process. It involves massive early cell death in most of the bulge followed by its repopulation by hair

germ cells and concomitant new hair regeneration cycle (Ito et al. (2002) J Invest Dermatol. 119:1310-1316). Mechanism of plucking-induced regeneration, however, is not understood. If plucking induces regeneration via an intrinsic mechanism than plucking of one hair should always lead to the regeneration of one injured follicle. Although this is true for anagen vibrissa follicles, this was intrigued by clear doze-dependent regenerative response of telogen pelage (body) follicles. Plucking of 200 neighboring hairs leads to significantly faster regeneration than plucking of 50 hairs, while plucking of just 20 hairs almost never elicits regenerative response (Plikus et al. (2008) Nature 451:340-344). This study investigates this phenomenon further to understand the role of inter-follicular dermal macro-environment in reparative regeneration of hair stem cells and to elucidate molecular signaling involved.

Quantitative plucking reveals hair regeneration to be a population behavior.

[0210] To understand how inter-follicular macro-environment influences regenerative response of hair follicles to plucking, this example designed "plucking density" experiment. In it 200 telogen hairs were evenly plucked from circular area of the skin, so that density of plucking-injured follicles decreased as the diameter of the area increased. To standardize this experiment, this example modified the inventors' original protocol (Plikus et al. (2008) Nature 451:340-344) and performed plucking 21 days after waxing-induced hair cycle synchronization, when all hair follicles were in telogen. When plucked at 100% density, 200 hairs occupy 1.44π mm^2 of skin surface (2.4 mm in diameter), which means that the hair density in dorsal skin of adult mice is approximately $139/\pi$ ($hair/mm^2$). Follicles within areas plucked at 100% density re-enter anagen after 12 days (Fig. 1A). However, when 200 hairs were plucked at low density of 2 $hair/mm^2$ (evenly over 100 mm^2 of skin surface), no hair regeneration was observed even after 30 days. This phenomenon of plucking density-dependent hair regeneration unequivocally indicate that hair stem cells respond to follicular injury indirectly and likely require additional dose-dependent signal from dermal macro-environment. To examine density-dependent phenomenon further and to find the minimal plucking density required to generate above-threshold signaling 200 hairs were plucked from different circular skin surface areas: 2.25π (3mm in diameter); 4π (4mm in diameter); 6.25π (5mm in diameter); 9π (6mm in diameter); 12.25π (7mm in diameter) and 16π (8mm in diameter). These experiments resulted in the plucking densities of $89/\pi$, $50/\pi$, $32/\pi$, $22/\pi$, $16/\pi$ and $12.5/\pi$ ($hair/mm^2$)

respectively. Their results show that hair regeneration occurs only when plucking density is higher than $32/\pi$ (Fig. 1B, 1D, 5). Importantly, the number of follicles that regenerated from 2.25π, 4π and 6.25π plucked areas were 452, 776.8 and 1276 respectively (Fig. 1C). In case of 6.25π area, all 868 follicle within the boundaries of plucked area regenerated (i.e. 4 times more than number of plucked follicles) (circle, Fig. 1E). In addition, through hair wave propagation, the surrounding follicles outside the boundaries of plucked area were also induced to regenerate (circle, Fig. 1E). Thus, when plucking is done at over-threshold density ($32/\pi$ hair/mm^2), regenerative response is not restricted to plucked follicles, but also occurs in surrounding, non-plucked follicles. This result confirms existence of dose-dependent macro-environmental signal and shows that regenerative response of telogen follicles to this signal is independent of plucking injury *per se*. Without being bound by theory, Applicants hypothesize that a certain population of cells in telogen follicles senses and responds to mechanical injury produced by plucking. In response to injury, each follicle releases a "quantum" of first-level activating signal into the dermal macro-environment. Identity of these cells and first-level activating signal are currently unknown. Further, Applicants propose that this initial signal stimulates one or several cell types in dermal macro-environment to secrete second-level paracrine activator(s) that reciprocally signal back to the neighboring telogen follicles (both plucked and non-plucked) and induce them to regenerate. Only when enough macro-environmental activatorsare accumulated in certain area does the cumulative activating signal reach the "threshold" level and result in hair regeneration.

Identifying novel macro-environmental modulators

[0211] To test the hypothesis on the involvement of macro-environmental signaling Applicants performed differential expression profiling for candidate diffusible factors within and outside the areas of quantitative plucking. Candidate factor should become differentially upregulated within affected area soon after plucking, but prior to hair regeneration. Due to the important role of WNT signaling in hair regeneration (Lowry et al. (2005) Genes Dev 19:1596-1611; Enshell-Seijffers et al. (2010) Dev Cell 18:633-642), Applicants profiled expression of several WNT ligands, including *Wnt5* and *Wnt6* by whole mount *in situ* in the dorsal skin collected at different time points after plucking. *Wnt5* and *Wnt6* ligands were upregulated within new anagen follicles 4 days after plucking and remained expressed throughout the anagen phase (Fig. 2A). None of the

profiled WNT pathway members were differentially upregulated prior to anagen initiation, making WNT signaling an unlikely macro-environmental mediator of plucking-induced regeneration. Because plucking induces dramatic intra-follicular cell death prior to anagen initiation (Ito et al. (2002) J Invest Dermatol. 119:1310-1316), Applicants hypothesized that one or several diffusible pro-cell death cytokines can fulfill the role of macro-environmental modulator. Applicants examined various cytokines that can exhibit dual signaling properties (both pro-apoptotic and pro-proliferative) and identified TNF-α for its known ability to induce cell death and also activate NF-κB signaling (Van Antwerp et al. (1996) Genes Dev. 15:1219-1224; Wang et al. (1996) Science 275:784-787). Indeed, Applicants found that *TNF-α* and also pro-inflammatory cytokines *IL-1α*, *IL-1β* are markedly upregulated in dermal macro-environment just 2 days after plucking, ahead of anagen initiation, which occurs on day 4 (Fig. 2A). Except being a mediator for apoptotic process, NF-κB could also mediate Eda A1/EdaR signaling to activate Shh and cyclin D1 expression, and controls hair follicle development (Schmidt-Ullrich et al., 2006), so it is possible that Eda pathway may play an important role in plucking regeneration process. However, the expression of Eda was similar to Wnt signaling pathway, which was restricted inside the hair follicles without any extra-follicular distribution (Fig. 2A). Besides, Eda was activated at 4th day after plucking, which reduce the possibility that it could affect surrounding hair follicles to initiate anagen formation simultaneously. To figure the primary source of TNF-α, Applicants determine the expression pattern of macrophage and found it was upregulated 2 days after plucking and colocalized with TNF-α (Fig. 2A, 2B), which served as a proof that macrophage plays an important role in plucking induced anagen formation.

TNF-α mediates initiation of regeneration via NF-κB signaling

[0212] Potential stimulating effect of TNF-α, IL-1α, IL-1β on hair regeneration can be mediated via NF-κB signaling, which can enhance WNT signaling (Hyun et al. (2008) Biochim Biophy Acta 1783:419-428; Cawthorn et al. (2007) Cell Death and Differentiation 14:1361-1373; Kaler et al. (2009) Cancer Microenviron 2:69-80). Indeed, intra-cutaneous delivery of NF-κB inhibitor can delay plucking-induced hair regeneration by 10 days (Fig. 3A). FGF signaling pathway which served as a player in the initial step of hair regeneration (Greco et al. (2009) Cell Stem Cell 4:155-169) through FGFR2IIIb receptor to activate Ras and phosphorylating ERK was regulated by β-catenin (Enshell-

Seijffers et al. (2010) Dev Cell 18:633-642). Our data revealed all the inhibitors of FGF signaling pathway, including FGF receptor, Ras and ERK could delay plucking induced hair regeneration by 5 days, 13 days and 6 days independently (Fig. 3C, D, E). In contrary, TNF70-80 (TNF-α mimetic peptide) accelerates plucking-induced regenerative response (Fig. 3F). In addition, injection TNF70-80 coated beads into un-plucked telogen skin can elicit anagen re-entry directly (Fig. 4C). *TNF-α* null mice also exhibit a 10 days delay in regenerative response following plucking of 200 hairs (Fig. 4D). Besides, plucking 200 hairs from circular surface area with diameter of 3mm showed TNF-α could express not only below the hairs being plucked but also the hairs not being plucked (Fig. 4A) which also proves that the activators induced by plucking could affect the surrounding hair follicles not being plucked. In addition, the expression of BMP-2 is down regulated after 200 hairs are plucked from circular surface area with diameter of 3mm (Fig. 4B), which means that the refractory telogen was overcome and explained why the hairs from the whole area Applicants plucked could regenerate simultaneously. These data support the idea that hair plucking could turn on the expression of NF-κB through TNF-α due to apoptosis of keratinocytes and then NF-κB might induce anagen re-entry through WNT/β-catenin and FGF signaling pathway (Fig. 5).

[0213] A recent study revealed that the maintenance of telogen status is achieved by the expression of Bmp6/Fgf18 secreted by K6+ bulge cells that surround club hair structure. Upon plucking, quiescent signals (FGF18, BMP6) from K6+ bulge cells are lost, greatly reducing the threshold for anagen activation (Hsu et al., 2011). Without being bound by theory, Applicants submit that this intra-follicular regulation mechanism could only partially explained why plucking might result in hair regeneration. Applicants' prior research regarding original quantitative plucking experiments that showed clear dose-dependency of activating signal from the number of injured hair follicles (Plikus et al. (2008) Nature 451:340-344). If this model is correct, then plucking even one single hair will induce anagen in that follicle. In this disclosure, Applicants report the mechanism of reparative regeneration of hair follicles in response to plucking-induced injury. Surprisingly, Applicants found that although plucking produces intra-follicular injury, regenerative response is non-autonomous and involves reciprocal interactions between injured hair follicles and dermal macro-environment. Here Applicants demonstrate that relative topology of injured follicles is just as important. Because plucking of 200 hairs at different density shows clear density-dependent regenerative response, Applicants

concluded that not only the total number of injured follicles, but also their relative proximity influences strength of the activating signal. These findings allowed Applicants to hypothesize that dermal macro-environment that surrounds injured follicles is involved in generation of the activating signal.

[0214] It had been proved that inflammatory mediator TAK1, which results in activation of NF-κB through TNF-α could regulates hair follicle induction and morphogenesis, and is required for anagen induction and anagen maintenance (Sayama et al. (2010) PLoS One 5(6):e11275). Applicants profiled dermal macro-environment for the potential mediators and identified marked upregulation of several inflammatory cytokines in the plucked areas, most distinctly TNF-α. Because of this marked expression response, Applicants examined TNF-α signaling further, and functional experiments with TNF-α mimetic peptides and TNF-α null mice confirmed the important activating role macro-environmental TNF-α plays upon plucking-induced hair regeneration. Applicants show that macro-environmental TNF-α likely signals back to the telogen hair follicles via NF-κB pathway which ultimately leads to their regeneration. Importantly, plucking injury *per se* is not essential for priming telogen hair follicles for NF-κB-mediated response. In experiments with lower plucking density, both injured and un-injured telogen hair follicles regenerate over the entire plucked area. Applicants' data also demonstrated that WNT/β-catenin and FGF signaling are also involved in plucking induced hair regeneration. Though the relationships among NF-κB, WNT/β-catenin and FGF were unclear, however, according to previous studies which revealed that Tnf-α signaling can lead to the activation of canonical WNT pathway via NF-κB (Hyun et al. (2008) Biochim Biophy Acta 1783:419-428; Cawthorn et al. (2007) Cell Death and Differentiation 14:1361-1373), and *Wnt10a* and *Wnt10b* are known downstream NF-κB targets during hair follicle development (Zhang et al. (2009) Dev Cell 17:49-61), it is plausible that activation of hair follicles by NF-κB pathway includes one or several intermediate steps and involves WNT/β-catenin and FGF signaling events known to be critical for physiological anagen initiation (Greco et al. (2009) Cell Stem Cell 4:155-169; Enshell-Seijffers et al. (2010) Dev Cell 18:633-642). Therefore a model emerges, where:

(1) A hair plucking event is sensed by some cells of telogen follicle that in response to this physical injury signal to the surrounding dermal macro-environment and induces strong and localized expression of several inflammatory cytokines. Identity of

sensing cells and signaling pathway have yet to be determined.

(2) Concentration of macro-environmental cytokines and the reciprocal regenerative response to these cytokines by hair follicles is strongly dependent on the number and relative topology of plucked follicles. Only when enough activating cytokines in a certain area are accumulated and reach the "threshold" could they induce regenerative response.

(3) Once "threshold" macro-environmental signaling is obtained, all telogen follicles in the area (both plucked and non-plucked) can be induced to regenerate. Several macro-environmental modulators and several downstream signaling pathways are likely work simultaneously. Here Applicants identified novel TNF-α→NF-κB→WNT/FGF signaling axis that appears to distinguish injury-induced vs. physiological hair follicle regeneration (Plikus at al. (2008)) (*supra*).

[0215] These findings are important in understanding the mechanism of injury-induced hair regeneration and reveal that organ macro-environment is critical in regulating stem cell homeostasis. These results identify novel directions for studying reciprocal communication between stem cells and the larger niche. Shifting focus from hair follicle itself toward inter-follicular macro-environment may lead toward new approaches in treatment of various alopecias.

Methods

[0216] **Reagents:** NF-κB inhibitor (Bay11-7082) and FGFR inhibitor (PD173074) were purchased from Sigma. Ras inhibitor (Farnesylthiosalicylic acid) was purchased from Cayman Chemical. ERK inhibitor (U0126), JNK inhibitor (SP 600125), p38 inhibitor (SB 203580) and PI3K inhibitor (LY 294002) were purchased from Tocris Bioscience. (Ile^{76})-TNF-α (70-80)(human) was purchased from Bachem.

[0217] **Animals and surgical procedures:** 3-6 months C57BL/6 mice and TNF-null mice were used in this study. All procedures were performed on anaesthetized animals with protocols approved by USC vivaria. For hair plucking, 200 hairs were plucked from the following skin surface area: 1.44π (2.4mm in diameter); 2.25π (3mm in diameter); 4π (4mm in diameter); 6.25π (5mm in diameter); 9π (6mm in diameter); 12.25π (7mm in diameter); 16π (8mm in diameter) when the skin was in refractory telogen period.

[0218] Histology and molecular expressions: Tissues were collected, fixed, and processed for histology as described (Plikus et al., 2004). Whole mount *in situ* hybridization on thin slices of adult mouse skin were performed using the InsituPro automated *in situ* detection module (Intavis AG, Koeln, Germany). Analysis was performed according to the standard whole mount *in situ* protocol.

Example 2. Self-organizing and stochastic behaviors during the regeneration of hair stem cells

[0219] Stem cells cycle through active and quiescent states. Large populations of stem cells in an organ may cycle randomly or in a coordinated manner. Although stem cell cycling within single hair follicles has been studied, less is known about regenerative behavior in a hair follicle population. By combining predictive mathematical modeling with in vivo studies in mice and rabbits, Applicants show that a follicle progresses through cycling stages by continuous integration of inputs from intrinsic follicular and extrinsic environmental signals based on universal patterning principles. Signaling from the WNT/BMP activator/inhibitor pair is co-opted to mediate interactions among follicles in a population. This regenerative strategy is robust and versatile because relative activator/inhibitor strengths can be modulated easily, adapting the organism to different physiological and evolutionary needs.

Materials and Methods

[0220] Animals. C75BL/6J, SCID, KRT14-NOG (K14-Nog), KRT14-Wnt7a (K14-Wnt7a), BAT-gal, conductin-lacZ (cond-lacZ), cond-lacZ;K14-Wnt7a mice and New Zealand rabbits were studied.

[0221] Analysis of hair regeneration patterns. Hair regeneration patterns were monitored by periodically clipping fur and photographing the entire animal. Unlike plucking or shaving, the clipping of hair shafts does not affect physiological progression of the hair growth cycle and is non-invasive. Analysis of patterns was done by carefully studying skin pigmentation changes on timelines compiled from many sequential images of the same animal.

[0222] Transplantation procedures. All surgical procedures were performed on anesthetized animals following a protocol approved by USC animal resources. Skin transplantation was carried out when both donor rabbit and recipient SCID mouse skin

were in early telogen. This minimized the impact of wound healing on the hair growth cycle. After transplantation, recipient SCID mice were monitored for a prolonged period of time (~ one year). Changes in the hybrid hair regeneration patterns were documented and photographed.

[0223] Protein administration procedures. Intracutaneous administration of exogenous protein was performed as follows. Affinity chromatography Affi-gel blue gel beads were obtained from Biorad. Beads were washed in 1× PBS, followed by drying. The beads were then re-suspended in 5μl protein solution, either control (0.1% BSA) or experimental (recombinant mouse Wnt3a, 100ug/ml; recombinant mouse Dkk1, 100ug/ml), at 4 °C for 30 min. Both recombinant Wnt3a and Dkk1 were from R&D Systems. Reconstitution of the protein was as per the manufacturer's guidelines. Approximately 100 beads were introduced to the telogen skin of adult mice by means of a single puncture made by a 30G syringe needle. To replenish proteins, subsequent doses of 1.5 μl protein solution were microinjected to the site of the bead implantation every 24 hrs by means of a glass micro-needle. Subsequently skin was collected and inverted for whole-mount analysis of hair regeneration patterns around the control beads and beads with Wnt3a or Dkk1.

[0224] Analysis of whole-mount lacZ staining patterns. Skins from the entire body of *BAT-gal*, *cond-lacZ* and *cond-lacZ;K14-Wnt7a* mice were collected and whole-mount X-gal staining was performed. After staining, skin samples were de-hydrated in serial dilutions of EtOH and then re-hydrated. This was done to remove subcutaneous adipose tissue and expose telogen hair follicles for subsequent analysis and photography of lacZ staining patterns.

[0225] Predictive modeling of hair regeneration behavior. Applicants performed predictive mathematical modeling of the regeneration dynamics among hair stem cell clusters using a Cellular Automaton (CA) model. The patterning field is divided up into a number of square "cells" known as automata; they can be thought of as units which exhibit programmed responses to different signals. In this model, each automaton represents a single hair stem cell cluster (mouse, human) or all hair stem cell clusters of one compound follicle unit (rabbit) as it cycles through the following successive phases: signal propagating (***P***) and non-propagating phases (***A***), and phases refractory (***R***) and

competent (***C***) to such signals. Each automaton remains in a given phase for a variable interval of time after which it moves to the next state.

[0226] Applicants' CA model is constructed from a series of assumptions that are laid out below.

[0227] **Domain.** The domain is supposed to be rectangular and a grid is imposed on the domain with **nx** automata in a vertical direction and **ny** automata in a horizontal direction.

[0228] **Time.** Time is supposed to evolve in a series of discrete steps, each of equal length, **step**. The simulation runs until time **T** hours, hence using **T/step** steps in total (if the result is not an integer, then it is rounded up to the next time step).

[0229] **States.** Automata can be in one of four phases (*aka* steps):

- ***Competent (C)***: automata in this state are competent to enter the ***P*** state if triggered to do so.

- ***Propagating (P)***: automata in this state are able to influence surrounding ***C***-automata to enter ***P*** state.

- ***Autonomous (A)***: automata in this state do not influence surrounding automata and cannot be influenced themselves.

- ***Refractory (R)***: unlike ***C***-automata, automata in this state cannot be induced by ***P***-automata to enter ***P*** state.

[0230] Automata cycle through the states above. Times spent in the phases ***P***, ***A*** and ***R*** are distributed according to a Gamma distribution with specified mean and variance. The time spent in ***C*** is dependent on intrinsic and extrinsic signals. An automaton cannot re-enter ***P*** unless its neighboring environment displays certain behaviors: a supra-threshold stimulus is needed for the transition from ***C*** to ***P*** and the stimulus can arise in two different ways. Firstly, Applicants assume that ***C***-automata can spontaneously gain the ability to re-enter ***P*** and when they do so, they send out a stimulus. However, a sufficient number of neighboring automata must also spontaneously re-enter ***P*** to force the stimulus above threshold levels. Secondly, Applicants assume that automata in ***P*** send out stimuli: an automaton in ***C*** can respond to such signals, but a certain number of its neighbors must

be in ***P*** for an automaton to receive a large enough stimulus to re-enter ***P*** itself. If neither of the above criteria are satisfied then an automaton remains in ***C***.

[0231] Details of the algorithm are outlined in more detail below.

[0232] ***Competent (C).*** From this state, automata can enter ***P*** via one of two routes. The first depends on signals intrinsic to the automaton: at every time step, an automaton has a certain probability, **1-rnd_th**, of becoming able to spontaneously enter ***P***. It retains this ability for a period of time chosen from a Gamma distribution with mean **meanS** and standard deviation **sdS**. If at least **sp_th** of its neighbors also have this ability or are already in ***P***, then an automaton will enter ***P***. The second way in which an automaton may enter ***P*** is if it is induced to do so via external signals: if at least **p_th** of its neighbors are in ***P***, then an automaton enters ***P***. The definition of "neighbor" is an automaton lying in the Moore neighborhood – the eight surrounding automata.

[0233] ***Propagating (P).*** When automata in ***C*** receive a supra-threshold signal they enter ***P***, where they become able to influence other ***C***-automata. The length of time spent in this state is also randomly selected from a Gamma distribution, but with mean **meanP** and standard deviation **sdP**. After the specified time automata enter autonomous state (***A***).

[0234] ***Autonomous (A).*** When automata leave ***P*** they enter ***A***, and cannot be influenced by surrounding automata. The length of time spent in this state is also randomly selected from a Gamma distribution, but with mean **meanA** and standard deviation **sdA**. After the specified time automata enter the refractory state (***R***). If there is no autonomous state, **meanA** and **sdA** are set to zero and this phase is skipped.

[0235] **Refractory (*R*).** When automata leave ***A*** they enter ***R*** and cannot be influenced by surrounding automata. The length of time spent in this state is also randomly selected from a Gamma distribution, but with mean **meanR** and standard deviation **sdR**. After the specified time automata enter competent state (***C***) and the cycle begins once again.

[0236] **Gamma distribution.** The Gamma distribution is given by

$$f(x;k,\theta) = x^{k-1}\frac{e^{-x/\theta}}{\Gamma(k)\theta^{k}}$$

where k and μ are positive parameters. It has mean $\mu=k\theta$and variance $\sigma^2 = k\,\theta^2$. This implies for ***P***, for example, μ=**sdP**^{2}/**meanP** and k =**sdP**^{2}/**meanP**^{2}. Since we work with

discrete times, the length of time spent in each state is rounded to the nearest integer value.

[0237] **Updating.** At every time step the state of each automaton is updated: the update order is specified by creating a new random permutation of the automata at each step.

[0238] **Initial states.** Unless otherwise stated, all automata are initially taken to be in ***C***.

[0239] **Boundary conditions.** Absorbing boundary conditions are applied on each edge of the grid: an extra layer of automata is added to each edge and these automata are maintained in ***C***.

[0240] **Computation.** The procedure was implemented in C++ with each automaton represented by an object with two variables: the current state and the amount of time before the state was due to change. A class consisting of a two-dimensional array of objects was used to represent the field. This class contained parameter variables, the afore-mentioned field of "cells", a variable keeping track of the number of generations the field of "cells' had passed through, and the following groups of functions:

- functions to read/write to the automaton objects;
- functions that recorded the state of the entire field of automata;
- functions that checked and applied boundary conditions;
- functions that calculated the number of nearest neighbors of a particular state a given "cell" had;
- functions to measure and record the fractal dimension of the pattern present at a given generation;
- functions that read and adjusted the parameters;
- functions that updated the field according the model rules.

[0241] A set of utility functions was used to generate random calls from gamma and uniform distributions and to create ppm image files given rgb values. The movies were created by converting the ppm images to bmp images and using QuickTime software.

[0242] Parameters. The simulation parameters, their definitions and the values commonly used in numerical simulations are given in Table 1 below.

[0243] Data representation. The grid of automata states is plotted over time and the sequence of plots is converted to a movie file. Two types of plots are stored: the first shows each state as a different color (***P*** – blue, ***A*** – yellow, ***R*** – red, ***C*** – green) whilst the second shows expected pigmentation states as they would appear on the skin (***P*** – grey, ***A*** – black, ***R*** and ***C*** – white). In every simulation the state of the field was recorded at four-hour intervals and movies were created at 15 frames per second.

Table 1. Parameters used in the model simulations.

Parameter	Description	Balanced activator/inhibitor	Activators↑	Activators↑↑	Activators↑↑
Nx	Number of automaton rows	80	80	80	400
Ny	Number of automaton columns	80	80	80	400
step	Number of hours per time step	4 hours	4 hours	4 hours	4 hours
T	Total duration of simulation	100-200 days	100-200 days	100 days	100-300 days
meanP	Mean time spent in ***P***	4.0 days	4.0 days	14.0 days	30.0 days
sdP	Standard deviation of times spent in ***P***	0.5 days	0.5 days	0.5 days	0.5 days
meanA	Mean time spent in ***A***	10.0 days	10.0 days	0.0 days	0.0 days
sdA	Standard deviation of times spent in ***A***	1.0 days	1.0 days	0.0 days	0.0 days
meanR	Mean time spent in R	28.0 days	12.0 days	12.0 days	28.0 days
sdR	Standard deviation of times spent in ***R***	1.0-10.0 days	1.0-10.0 days	1.0 days	1.0-15.0 days
meanS	Mean time spent in ***C***	3.0 days	3.0 days	3.0 days	3.0 days
sdS	Standard deviation of times spent in ***C***	1.0 days	1.0 days	1.0 days	1.0 days
sp_th	Min. number of neighbors to spontaneously enter ***P***	4	4	2	4
p_th	Min. number of neighbors in P to stimulate entry to ***P***	3	3	2	3
r_th	Probability of becoming spontaneously able to enter ***P***	0.992-0.995	0.992-0.995	0.998-1	0.992-0.995

Table 2. Parameters used in the human model simulations.

Parameter	Description	Domain 1 (Fronto-parietal)	Domain 2 (Occipital)
nx	Number of automaton rows	150	
ny	Number of automaton columns	150	
step	Number of hours per time step	4 hours	
T	Total duration of simulation	1000 days	
sp_th	Min. number of neighbors to spontaneously enter ***P***	0	

p_th	Min. number of neighbors in P to stimulate entry to ***P***	3	
r_th	Probability of becoming spontaneously able to enter ***P***	0.998	
1st cycle			
meanP	Mean time spent in ***P***	5.0 days	5.0 days
sdP	Standard deviation of times spent in ***P***	0.5 days	0.5 days
meanA	Mean time spent in ***A***	65.0 days	135.0 days
sdA	Standard deviation of times spent in ***A***	0.5 days	0.5 days
meanR	Mean time spent in ***R***	28.0 days	98.0 days
sdR	Standard deviation of times spent in ***R***	0.5 days	0.5 days
meanS	Mean time spent in ***C***	3.0 days	3.0 days
sdS	Standard deviation of times spent in ***C***	1.0 days	1.0 days
2nd cycle			
meanP	Mean time spent in ***P***	5.0 days	5.0 days
sdP	Standard deviation of times spent in ***P***	0.5 days	0.5 days
meanA	Mean time spent in ***A***	44.0 days	79.0 days
sdA	Standard deviation of times spent in ***A***	0.5 days	0.5 days
meanR	Mean time spent in ***R***	92.0 days	84.0 days
sdR	Standard deviation of times spent in ***R***	0.5 days	0.5 days
meanS	Mean time spent in ***C***	3.0 days	3.0 days
sdS	Standard deviation of times spent in ***C***	1.0 days	1.0 days
3rd and following cycles			
meanP	Mean time spent in ***P***	0.0 days	
sdP	Standard deviation of times spent in ***P***	0.0 days	
meanA	Mean time spent in ***A***	84.0 days	
sdA	Standard deviation of times spent in ***A***	14.0 days	
meanR	Mean time spent in ***R***	84.0 days	
sdR	Standard deviation of times spent in ***R***	14.0 days	
meanS	Mean time spent in ***C***	3.0 days	
sdS	Standard deviation of times spent in ***C***	1.0 days	
Cycles upon alopecia			
meanP	Mean time spent in ***P***	0.0 days	
sdP	Standard deviation of times spent in ***P***	0.0 days	
meanA	Mean time spent in ***A***	7.0 days	
sdA	Standard deviation of times spent in ***A***	0.5 days	
meanR	Mean time spent in ***R***	336.0 days	
sdR	Standard deviation of times spent in ***R***	0.5 days	
meanS	Mean time spent in ***C***	3.0 days	
sdS	Standard deviation of times spent in ***C***	1.0 days	

[0244] To simulate human hair regeneration, the patterning field was divided into two domains: domain 1 (upper 2/3) representing the fronto-parietal scalp, and domain 2 (lower 1/3) representing the occipital scalp. Automata in each domain behave according to the parameters specified in the table above. These parameters are derived from known regeneration behavior of scalp hair follicles during 1st and 2nd fetal growth cycles (Cutrone Cutrone and Grimalt (2005) Eur J Pediatr 164:630; Halloy (2000) PNAS 97:8328), as well as during normal adult growth cycles and growth cycles upon alopecia.

Table 3. Percentage of telogen hair follicles (HFs) with spontaneous WNT activity in dermal papillae during *R*-phase and *C*-phase.

Mice	Solitary HFs	Groups of 2 HFs	Groups of 3 HFs	Groups of 4 HFs	Groups of 5+ HFs
BAT-Gal	0.18% 4.89%	0% 0.23%	0% 0.03%	0% 0.01%	0% <0.01%
Cond-lacZ	0.10% 4.98%	0% 0.16%	0% 0.06%	0% 0.02%	0% <0.01%
Cond-lacZ; K14-Wnt7a	~100% WNT-active ~100% WNT-active				

Results

[0245] Continuous stem cell (SC) regeneration is essential for the maintenance of many adult organs, for example, in the bone marrow, skin, and gastrointestinal tract. Although regenerative behavior within a single SC cluster such as the hair bulge (Fuchs (2009) Cell Stem Cell 4:499) or intestinal villi (Li and Clevers (2010) Science 327:542) has been studied, it is largely unknown how the regenerative behavior in populations of these SC clusters is coordinated. During development, thousands of cells can self-organize into anatomic structures and patterns by coordinating just a few morphogenetic signals (Chuong and Richardson (2009) Int J Dev Biol 53:653), as seen in the periodic patterning of skin appendages (Jiang et al. (1999) Development 126:4997; Sick et al. (2006) Science 314:1447). Without being bound by theory, Applicants hypothesize that the regenerative cycling of adult organ SCs can be similarly coordinated by diffusible signals and self-organize into spatiotemporal regenerative patterns.

[0246] Hair offers a suitable experimental model because hair follicles (HFs) cycle through phases of growth (anagen) and rest (telogen) (Stenn and Paus (2001) Physiol Rev 81:449). SCs are clustered in hair bulges, making them easier to study than SCs in other

organs where they are usually scattered randomly (Morris et al. (2004) Nat Biotechnol 22:411) (Fig. 10A). Growing hairs produce pigmentation patterns that allow simultaneous monitoring of the regenerative behavior of thousands of SCs (Fig. 6A) (Suzuki et al. (2003) PNAS 100:9680; Plikus and Chuong (2008) JID 128:1071). Additionally, the skin is flat, restricting interactions to two dimensions, further simplifying the analysis.

[0247] Applicants developed a Cellular Automaton (CA) model consisting of a regular grid of automata with one automaton representing one HF (Fig. 10B). The eight automata surrounding one automaton are defined as its neighbors. With time, the state of each automaton changes according to rules that take into account the state of neighboring automata. Automata in certain states can interact, generating complex, self-organizing patterns based on a simple set of rules. Such patterning behavior can be globally modulated by simple rule changes in local automaton-to-automaton interactions (Wolfram (2002) A new kind of science).

[0248] To form regenerative patterns, activating signals among SCs should be able to spread and stop. This is possible when SCs can differentially respond to the same signal at different times of their regenerative cycle. Applicants previously identified four functional phases in the hair regenerative cycle: signal propagating (*P*) and non-propagating phases (*A*), and phases refractory (*R*) and competent (*C*) to such signals (Plikus et al. (2008) Nature 451:340). Telogen HFs in *R*-phase cannot enter anagen because BMPs in the surrounding skin macro-environment keep hair SCs quiescent (Plikus et al. (2008) Nature 451:340). Telogen HFs in *C*-phase are devoid of these inhibitors and can enter anagen as long as the sum of intrinsic and extrinsic activators is above the threshold. Intrinsic activators are produced as the result of SC and dermal papilla interactions. Extrinsic activators come from neighboring *P*-phase anagen HFs, and represent a form of collective positive feedback. Thus, HFs can enter anagen in two ways: autonomously depending on the level of intrinsic activation, or non-autonomously, when activators are delivered by the surrounding macro-environment. The probability of anagen entry is based on the sum of these fluctuating inputs.

[0249] Here, Applicants use mathematical simulations to test the sufficiency and robustness of this model. We show that the CA model encompassing $P \rightarrow A \rightarrow R \rightarrow C$ cycling can reproduce the full spectrum of hair regenerative patterns observed in mice:

formation of initiation centers, wave spreading, maintenance of borders and border instability (Fig. 6B, and data not shown; Table 1).

[0250] Applicants have also shown that predictive modeling of the regeneration dynamics among hair stem cell clusters with balanced activator/inhibitor levels, each showing spreading of a regenerative wave, multiple initiation events, borders can be maintained over several cycles and Border instability.

[0251] For a model to be robust and capture conserved patterning principles, it should be capable of explaining the diverse regenerative patterns seen in mutant mice and other animals. The duration of each phase of the regenerative cycle depends on the relative strengths of activators and inhibitors (Fig. 6A). Without being bound by theory, Applicants suggest that diffusible signaling molecules used for regulating SC activities within the HF are co-opted to mediate interactions between neighboring HFs. Activator-driven propagation of regenerative waves and inhibitor-driven halting of wave propagation can be potentiated by respective ligands or dampened by antagonists secreted between HFs by the skin macro-environment. Together, HF and skin macro-environment derived ligands and antagonists should combine to produce unique signaling profiles that define properties of $P \rightarrow A \rightarrow R \rightarrow C$ phases. (Data not shown). Applicants assigned generic signaling profiles for each of the four phases in terms of the activator/inhibitor ratio: *P*-high/low; *A*-high/high; *R*-low/high and *C*-low/low (Fig. 6A). Applicants undertook predictive modeling using their CA framework to anticipate how regenerative patterns might be altered by changing the level of hypothetical activators or inhibitors (Fig. 6B, and data not shown; Table 1). The key prediction from this model is that the strength of SC coupling can be weakened by either inhibitory pathway ligands or activating pathway antagonists.

[0252] Applicants also have predictive modeling of the regenerative patterns upon progressive increase in activator levels. The CA model predicts faster global regeneration dynamics when parameters of automaton cycle are adjusted to reflect a progressive rise in activator: duration of R-phase is shortened; duration of R-phase is shortened, duration of P-phase is increased at the expense of A-phase, minimal number of neighboring automata required to spontaneously enter P-phase is reduced and probability of spontaneous P-phase entry is increased. Control simulations performed with balanced activator/inhibitor levels.

[0253] Indeed, previously Applicants showed that inhibitory BMP pathway ligands produced by the skin macro-environment maintain telogen HFs in *R*-phase and prevent them from being activated by the advancing regenerative wave (Plikus et al. (2008) Nature 451:340). To identify activating pathway(s) involved in SC coupling, Applicants began by matching expression patterns of various diffusible antagonists with the signature pattern predicted by the CA model: *P*-low; *A*-high; *R*-high and *C*-low. *Dkk1* and *Sfrp4*, both diffusible WNT pathway antagonists, prominently fit this expression pattern (Fig. 7B, and data not shown). Simultaneously, multiple WNT ligands are expressed by anagen HFs (data not shown) (Reddy et al. (2001) Mech Dev 107:69), and, in principle, can serve as diffusible activators to mediate regenerative coupling between HFs. This scenario predicts that competing WNT ligands and antagonists produce distinct patterns of WNT signaling: WNT is activated in *C*-phase telogen HFs adjacent to *P*-phase anagen HFs, but not in the same *C*-phase HFs next to *A*-phase anagen HFs.

[0254] To gauge WNT signaling Applicants used *BAT-gal* and *cond-lacZ* WNT-reporter mice, where lacZ expression marks canonical WNT pathway activation. Indeed, WNT-reporter mice show these predictions to be true. Massive WNT pathway activation is seen in *C*-phase HFs ahead of the *P*→*C* regenerative wave-front (data not shown), but not along the *A*-*C* border (data not shown). According to CA model predictions, the activating pathway should demonstrate stochastic signaling in *C*- but not *R*-phase HFs, forming the basis for rare spontaneous *C*→*P* activation events. Applicants found striking differences in WNT signaling between *R*- and *C*-phases. Although there is virtually no WNT activation in *R*-phase, both reporter mice show many stochastically distributed WNT-active telogen HFs in *C*-phase (Fig. 7E, and data not shown). The majority of WNT-active HFs are solitary (4.9% in *BAT-gal*, 5% in *cond-lacZ*) but groups of two to four WNT-active HFs are very rare (0.23-0.01%) (Table 3). This WNT activity is localized not to SCs, but to adjacent dermal papilla (DP) (data not shown). Applicants found that 99.9% of all spontaneous WNT activation events in DPs do not translate into spontaneous anagen initiations. However, when WNT activation occurs in groups of five or more DPs simultaneously (<0.01%), it leads to new anagen activation (data not shown). This illustrates the stochastic nature of activation events predicted by the model and implies that HFs work synergistically towards successful anagen reentry.

[0255] Applicants also have shown that predictive large-scale modeling of the regenerative patterns upon increase in activator levels. Multiple spontaneous *C→P* initiation events occur and multiple continuous *C→P* spreading waves exist simultaneously. As the simulation time increases, complex, fractal-like patterns develop.

[0256] Applicants have further provided model simulations of human scalp hair regenerative patterns. Modeling performed using a series of assumptions derived from known human scalp hair regenerative behavior during the first and second fetal growth cycles (Cutrone and Grimalt (2005) Eur J Pediatr 164:630; Halloy (2000) PNAS 97:8328), as well as during normal adult growth cycles and growth cycles upon alopecia can reproduce human scalp hair regenerative patterns.

[0257] Applicants further tested the functional role of WNT signaling locally with protein-coated beads and globally with transgenic mice. Local injection of Wnt3a beads induces new regenerative waves, whereas injection of Dkk1 beads disrupts advancement of the existing regenerative wave (Fig. 7C, and data not shown). Furthermore, when Wnt7a is over-expressed in *K14-Wnt7a* mice, there is shortening of *R*-phase (from 28 to 12 days) and enhanced SC activation, evidenced by many more spontaneous anagen initiation events and faster wave spreading compared to WT (Fig. 7A, and data not shown). In *cond-lacZ;K14-Wnt7a* mice, nearly 100% of DPs become WNT-active in *C*- and even *R*-phases (Fig. 7F), but no anagen reentry is observed in early *R*-phase. A simultaneous decrease in inhibitory BMP signaling upon an *R→C* transition is essential for WNT-mediated anagen reentry.

[0258] By fulfilling multiple CA model predictions for a hypothetical activator, WNT signaling emerges as the key pathway for mediating regenerative coupling between hair SCs. This shows that successful anagen reentry requires both down-regulation of BMP in the macro-environment and spontaneous up-regulation of WNT activity in DPs (data not shown). (1) Loss of inhibitors (BMP ligands and WNT antagonists) from the skin macro-environment in *C*-phase enables HFs to express fluctuating spontaneous WNT activity in DPs. (2) Such WNT activity in a single HF is not sufficient to drive anagen reentry. When several DPs adopt a WNT-active status simultaneously, they re-enforce each other and *C→P* activation occurs stochastically. Indeed, WNT signaling in DPs was recently shown to be critical for their anagen-inducing effect, likely mediated by secondary FGF

signaling (Enshell-Seijffers et al. (2010) Dev Cell 18:633). Alternatively, in regenerative waves the wave-front carries many activators that induce all *C*-phase HFs to enter anagen.

[0259] Compared to mice, rabbits have more robust hair growth. They have compound HFs (data not shown) (Whiteley (1958) Nature 181:850), each containing multiple tightly packed SC clusters and DPs (Fig. 8B-E, and data not shown). The skin surface area of rabbits is also 30 times larger than in mice. Applicants wanted to examine regenerative patterns in rabbits and see how our CA model fares against experimental data. Applicants observe that $C \rightarrow P$ activations in all SC clusters within one compound HF are closely coupled, and in the context of our CA model they behave as one "super-cluster" (one automaton) of SCs. Rabbits display complex, fractal-like regenerative patterns (Fig. 8A, 8F, and data not shown) closely reminiscent of patterns generated by our model (data not shown). By exploring their fractal geometry we show that large pieces of regenerative patterns in rabbits remain geometrically similar to much smaller pieces of themselves (data not shown). Thus, the same CA principles are effective in managing regeneration of hair SCs regardless of their total number, arrangement, or organ size. Notably, pattern-forming signaling cues are likely conserved between mice and rabbits, as activation events readily spread from rabbit to mouse HFs in the cross-species grafting system, resulting in hybrid patterns (data not shown).

[0260] Applicants' modeling predicts that significant increases in inhibiting signals or decreases in activating signals would reduce and eventually prevent coupling among HFs (Fig. 6B). Applicants observe see this in humans, where regenerative waves are observed in fetal scalp during the first two cycles (Cutrone and Grimalt (2005) Eur J Pediatr 164:630), but disappear in the adult when all HFs cycle independently and randomly (Halloy et al. (2000) PNAS 97:8328). In adults, the lack of SC coupling makes hair regeneration depend solely on intrinsic activation mechanisms, making it particularly vulnerable to any decreases in intrinsic SC activation. Without coupling, such decreases can ultimately lead to baldness as seen in androgenic alopecia and "short anagen syndrome" (data not shown) (Antaya et al. (2005) J Am Acad Dermatol 53:S130).

[0261] In summary, Applicants show for the first time how organ-wide SC management can be achieved. Applicants speculate that during evolution integration of signals from single to multiple hair follicles across skin likely facilitated the formation of new mechanisms of regeneration. SC clusters can now be regulated as one entity, allowing

organ regeneration to occur episodically with an intrinsic rate (data not shown). Co-option of key WNT and BMP signaling pathways from HFs by the skin macro-environment allows for coupling between the SC clusters (data not shown). Applicants conjecture that such a mechanism provides animals with a simple, yet robust and effective way to coordinate the regeneration of very large SC populations, otherwise impossible with an intrinsic activation mechanism alone. Applicants observe that regenerative hair patterns can differ in the same animal under different physiological conditions, allowing organisms to adapt to the environment (e.g., pregnancy in mice) (Plikus et al. (2008) Nature 451:340). At the evolutionary scale, macro-environmental regulation makes hair growth a highly modulatable trait. Finally, beyond HFs, the experimental accessibility of this system offers an excellent model for analyzing the fundamental principles of self-organizing behaviors in biological systems composed of coupled cycling elements.

[0262] It is to be understood that while the disclosure has been described in conjunction with the above embodiments, that the foregoing description and examples are intended to illustrate and not limit the scope of the disclosure. Other aspects, advantages and modifications within the scope of the disclosure will be apparent to those skilled in the art to which the disclosure pertains.

CLAIMS

1. A method for facilitating hair growth in a tissue containing a hair follicle comprising administering to the tissue containing a primed inter-follicular macro-environment, two or more agents selected from the group (a) a Bone Morphogenic Protein (BMP) signaling inhibitor, (b) a secreted frizzled-related protein 4 (Sfrp4) or dickkopf homolog 1 (Dkk1) inhibitor, (c) an NF-κB agonist, (d) a TNF-alpha, IL-1alpha or IL-1beta or an equivalent thereof, or (e) Follistatin, thereby facilitating hair growth.

2. A method for treating alopecia in a subject having tissue containing a hair follicle, comprising administering to the tissue containing a primed inter-follicular macro-environment, two or more agents selected from the group (a) a Bone Morphogenic Protein (BMP) signaling inhibitor, (b) a secreted frizzled-related protein 4 (Sfrp4) or dickkopf homolog 1 (Dkk1) inhibitor, (c) an NF-κB agonist, (d) a TNF-alpha, IL-1alpha or IL-1beta or an equivalent thereof, or (e) Follistatin, thereby treating alopecia in the subject.

3. The method of claim 1 or 2, wherein the two or more agents comprise (a), (b) and (e).

4. The method of claim 1 or 2, wherein the two or more agents comprise (c) and (d).

5. The method of claim 1 or 2, wherein the two or more agents comprise (a) and (c).

6. The method of any preceding claims, wherein the BMP inhibitor is one or more of dorsomorphin, noggin, chordin, gremlin or sclerostin.

7. The method of any of claims 1 to 5, wherein the BMP inhibitor is a small interference RNA (siRNA) or double strand RNA (dsRNA) that inhibits one or more genes selected from the group consisting of BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 and BMP15.

8. The method of any of claims 1 to 5, wherein the agent is an antibody or modified antibody that inhibits a BMP antagonist or activates or stabilizes a BMP protein is one or more of BMP1, BMP2, BMP3, BMP4, BMP5, BMP6, BMP7, BMP8a, BMP8b, BMP10 or BMP15.

9. The method of any of the preceding claims, wherein the two or more agents are administered topically.

10. The method of claim 9, further comprising administration of penetration enhancer prior to or concomitantly with administration of the two or more agents.

11. The method of any of the preceding claims, wherein the subject is a human patient.

PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13*ter*.1(c) and (d) and 39)

Applicant's or agent's file reference	**IMPORTANT DECLARATION**	Date of mailing (*day/month/year*)
064189-4611		01 AUGUST 2012 (01.08.2012)
International application No.	International filing date (*day/month/year*)	(Earlist) Priority date *(day/month/year)*
PCT/US2012/030765	**27 MARCH 2012 (27.03.2012)**	28 MARCH 2011 (28.03.2011)

International Patent Classification (IPC) or both national classification and IPC

A61K 38/18(2006.01)i, A61K 38/17(2006.01)i, A61K 48/00(2006.01)i, A61K 8/64(2006.01)i, A61K 31/7105(2006.01)i, A61P 17/14(2006.01)i, A61Q 7/00(2006.01)i

Applicant

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This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☒ The subject matter of the international application relates to:
   - a. ☐ scientific theories.
   - b. ☐ mathematical theories.
   - c. ☐ plant varieties.
   - d. ☐ animal varieties.
   - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes.
   - f. ☐ schemes, rules or methods of doing business.
   - g. ☐ schemes, rules or methods of performing purely mental acts.
   - h. ☐ schemes, rules or methods of playing games.
   - i. ☒ methods for treatment of the human body by surgery or therapy.
   - j. ☐ methods for treatment of the animal body by surgery or therapy.
   - k. ☐ diagnostic methods practised on the human or animal body.
   - l. ☐ mere presentation of information.
   - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art.
2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:

   ☐ the description ☐ the claims ☐ the drawings
3. ☐ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:
   - ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
   - ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
   - ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b)
4. Further coments:

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